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**FOR**

**METHODS AND COMPOSITIONS IN BREAST CANCER DIAGNOSIS  
AND THERAPEUTICS**

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## METHODS AND COMPOSITIONS IN BREAST CANCER DIAGNOSIS AND THERAPEUTICS

[0001] This application claims priority to USSN 60/304,018, filed July 9, 2001, and USSN 60/262,990, filed January 19, 2001.

[0002] This invention was developed with funds from the United States Government. The United States Government may have certain rights in the invention.

### FIELD OF THE INVENTION

[0003] The present invention is directed to the fields of cancer and molecular genetics. Specifically, the present invention is directed to the determination of susceptibility to breast cancer and the diagnosis of invasive breast cancer. More specifically, the present invention is directed to a mutation in estrogen receptor alpha (ER) and its association with breast cancer.

### BACKGROUND OF THE INVENTION

[0004] Invasive breast cancer (IBC) is one of the most common and lethal malignant neoplasms affecting women, especially in Western cultures. The majority of IBCs are thought to develop over long periods of time from certain preexisting benign lesions. There are many types of benign lesions in the human breast, and only a few appear to have significant premalignant potential. The most important premalignant lesions recognized today are referred to as atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS). Although DCIS and LCIS possess some malignant properties, such as loss of growth control, they lack the ability to invade and metastasize and, in this sense, are premalignant.

[0005] A skilled artisan is aware that investigation of the role of the estrogen receptor in carcinomas is described by Watts *et al.*, J. Steroid Biochem. Molec. Biol. 41(3), 529 (1992); Scott *et al.*, J. Clin. Invest. 88, 700 (1991); Ince *et al.*, J. Bio. Chem. 268, 14026 (1993); Fuqua *et al.*, Can. Res. 52, 43 (1992); McGuire *et al.*, Mol. Endocr. 5, 1571 (1991); Castles *et al.*, Can. Res. 53, 5934 (1993); and Weigel and deConinck, Can. Res. 53, 3472 (1993). Furthermore, description of the estrogen receptor mRNA may be found in Keaveney *et al.*, J. Mol. Endocr. 6, 111 (1991); Green *et al.*, Nature 320, 134 (1986); White

*et al.*, Mol. Endocr. 1, 735 (1987); and Piva *et al.*, J. Steroid Biochem. Molec. Biol. 46, 531 (1993).

[0006] U.S. Patent No. 6,162,606 is directed to identification of defective estrogen receptors associated with the classification of breast tumors which are responsive to or resistant to hormone therapy. Similarly, U.S. Patent No. 5,563,035 regards monitoring the level of ERF-1, a transcriptional regulator of expression of the estrogen receptor, as being indicative of the response of a breast tumor to various therapies.

[0007] There is epidemiological evidence that there are genetic alterations that are closely associated with morphological tumor progression, such as is found in studies in colon carcinoma (Vogelstein and Kinzler, 1993). In this model (Dupont and Page, 1985), breast cancer is hypothesized as evolving from normal ductal epithelium to typical hyperplasia, to atypical hyperplasia, to carcinoma *in situ*, to invasive carcinoma, and finally to metastatic carcinoma. Recent data also suggests that the majority of hyperplasias share molecular alterations with invasive disease in the same breast (O'Connell *et al.*, 1998), providing genetic evidence that they are related. Unlike colon cancer, very little is known about the specific molecular changes that are associated with the earliest stages of breast cancer evolution. However, it is likely that estrogens are important, since they are potent mitogens for normal breast epithelial cells, and it is believed that the duration of estrogen exposure to the breast epithelium is a significant risk factor for breast cancer development. It is also generally agreed that expression of the estrogen receptor (ER) is relatively low in normal breast epithelium, but is higher in certain premalignant lesions (*e.g.* typical hyperplasias) (van Agthoven *et al.*, 1994).

[0008] Anandappa *et al.* (2000) detected no sequencing variants, such as single base change mutations, in ER from a panel of human primary breast cancer specimens. However, Zhang *et al.* (1997) identified an ER mutant in metastatic breast cancer which had a constitutive transactivation function independent of estradiol-binding.

[0009] Current human breast cancer management strategies utilize ER status as a predictive factor (McGuire, 1978; Burstein, 1982; Brooks *et al.*, 1980; Degenshein *et al.*, 1980; McGuire *et al.*, 1975; McGuire, 1987; Elledge and McGuire, 1993; Gelbfish *et al.*, 1988; Williams *et al.*, 1987; Kohail *et al.*, 1985; Donegan, 1992; Millis, 1980; McCarty *et al.*, 1980), although none regard the specific mutation of the present invention. Present human breast tumor tissue specimens are subjected to both ligand-binding studies and immunohistochemical analyses to determine ER status (King *et al.*, 1979; Shousha *et al.*, 1989; Shousha *et al.*, 1990). Thus, as has been acknowledged (see, for example, Roger *et al.*,

2000), the art presently lacks a molecular marker for breast tissue, such as a premalignant lesion, which is at risk for breast cancer, particularly for invasive breast cancer, and also lacks a marker for the purpose of improving approaches to risk prediction and treatment strategies. Identification of a specific molecular marker for an altered ER as an early event in breast cancer evolution would be a significant advance in the field and would provide an ideal diagnosis tool for the detection of susceptibility to breast cancer and its subsequent prevention.

### SUMMARY OF THE INVENTION

[0010] In an embodiment of the present invention there is an isolated estrogen receptor alpha nucleic acid sequence comprising an A908G mutation.

[0011] In another embodiment of the present invention there is an isolated estrogen receptor alpha amino acid sequence comprising a K303R substitution.

[0012] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual, wherein the sample comprises a cell having an estrogen receptor alpha nucleic acid sequence; and assaying the nucleic acid sequence for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer. In a specific embodiment, the sample is from a premalignant lesion of the breast.

[0013] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of invasive breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; and assaying an estrogen receptor alpha nucleic acid sequence from a cell of the sample for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence detects susceptibility of the premalignant lesion to develop into the invasive breast cancer. In a specific embodiment, the sample is from a premalignant lesion of the breast.

[0014] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of invasive breast cancer from a premalignant lesion in a breast, comprising the steps of obtaining a sample from the premalignant lesion; dissecting the sample to differentiate hyperplastic cells in the sample from nonhyperplastic cells; and assaying an estrogen receptor alpha nucleic acid sequence from the hyperplastic cell of the sample for an A908G mutation, wherein the presence of the mutation in the

nucleic acid sequence detects susceptibility of the premalignant lesion to develop into the invasive breast cancer. In a specific embodiment, the dissection step comprises removal of the hyperplastic cells from the sample by manual manipulation or by laser capture microdissection. In another specific embodiment, the sample is obtained by biopsy. In a specific embodiment, the assaying step comprises sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection, or a combination thereof. In an additional specific embodiment, the assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or is by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence.

[0015] In an additional embodiment of the present invention there is a method of classifying breast cancer in an individual, comprising the steps of obtaining from the individual a sample from the breast, wherein the sample contains a cancer cell; and assaying an estrogen receptor alpha nucleic acid sequence from the cell of the sample for an A908G mutation, wherein the presence of the mutation identifies the breast cancer to be invasive breast cancer. In a specific embodiment, the sample is obtained by biopsy. In another specific embodiment, the assaying step is selected from the group consisting of sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection, and a combination thereof. In an additional specific embodiment the assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence.

[0016] In another embodiment of the present invention there is a method of diagnosing breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual, wherein the sample comprises a cell having an estrogen receptor alpha nucleic acid sequence; and assaying the nucleic acid sequence for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer.

[0017] In another embodiment of the present invention there is a method of diagnosing breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; dissecting the sample to differentiate a cell suspected of being cancerous from a noncancerous cell; and assaying the cell suspected of being cancerous for an A908G mutation in an estrogen receptor alpha nucleic acid sequence, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer. In a specific embodiment, the dissection step comprises removal of the cells

suspected of being cancerous from the sample by manual manipulation or by laser capture microdissection. In a specific embodiment, the sample is obtained by biopsy. In another specific embodiment, the assaying step is selected from the group consisting of sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection, and a combination thereof. In an additional specific embodiment, the assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or is by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence.

[0018] In another embodiment of the present invention there is a kit for diagnosing an A908G mutation in an estrogen receptor alpha nucleic acid sequence, comprising at least one primer selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:33, SEQ ID NO:34, and SEQ ID NO:35. In one embodiment, the primers are extendable. In an alternative embodiment, the primers are nonextendable.

[0019] In another embodiment of the present invention there is a monoclonal antibody that binds immunologically to an acetylated estrogen receptor alpha amino acid sequence, or an antigenic fragment thereof.

[0020] In another embodiment of the present invention there is a monoclonal antibody that binds immunologically to an A908G mutation in an estrogen receptor alpha nucleic acid sequence.

[0021] In an additional embodiment of the present invention there is a method to correct a G mutation at nucleotide 908 of an estrogen receptor alpha nucleic acid sequence in a cell of an individual, comprising the step of administering to the cell an estrogen receptor alpha nucleic acid sequence comprising an A at nucleotide 908. In a specific embodiment, the estrogen receptor alpha nucleic acid sequence comprising an A at nucleotide 908 is present on a vector. In another specific embodiment, the vector is selected from the group consisting of plasmid, viral vector, liposome, and a combination thereof. In an additional specific embodiment, the viral vector is selected from the group consisting of adenoviral vector, retroviral vector, adeno-associated viral vector, or a combination thereof.

[0022] In an additional embodiment of the present invention there is a method to prevent breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; identifying in the sample an A908G mutation in a nucleic acid sequence of estrogen receptor alpha; and correcting the A908G mutation, wherein the correction results in the prevention of the breast cancer. In a specific embodiment, the breast

sample is from a premalignant lesion of the breast. In another specific embodiment, the correction step comprises administering an estrogen receptor alpha nucleic acid sequence comprising a G at nucleotide 908 to a cell comprising an estrogen receptor alpha nucleic acid sequence containing the A908G mutation.

[0023] In an additional embodiment of the present invention there is a method to treat breast cancer in an individual, wherein an estrogen receptor alpha nucleic acid sequence in a breast cell of the individual has an A908G mutation, comprising the step of administering to the cell an estrogen receptor alpha nucleic acid sequence comprising a G at nucleotide 908.

[0024] In another embodiment of the present invention there is a method to prevent breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; identifying in the sample an arginine at amino acid residue 303 in an amino acid sequence of estrogen receptor alpha; and administering to the individual an amino acid sequence of estrogen receptor alpha comprising a lysine at amino acid residue 303, wherein the administration results in the prevention of the breast cancer. In a specific embodiment, the breast sample is from a premalignant lesion of the breast.

[0025] In an object of the present invention there is a method of identifying a modulator of an estrogen receptor alpha K303R polypeptide, comprising providing a candidate modulator; admixing the candidate modulator with an isolated compound or cell, or a suitable experimental animal; measuring one or more characteristics of the compound, cell or animal; and comparing the characteristic measured with the characteristic of the compound, cell or animal in the absence of the candidate modulator, wherein a difference between the measured characteristics indicates that the candidate modulator is the modulator of the compound, cell or animal.

[0026] In another object of the present invention, there is a method of screening for a modulator of an estrogen receptor alpha polypeptide comprising a K303R substitution, comprising introducing to a cell a vector comprising a nucleic acid sequence which encodes the estrogen receptor alpha K303R polypeptide; a vector comprising at least one estrogen-responsive regulatory element operatively linked to a reporter polynucleotide; and a test agent; and assaying expression of the reporter polynucleotide in the presence of the test agent, wherein the test agent is the modulator when the reporter polynucleotide expression changes in the presence of the test agent. In a specific embodiment, at least one of the vectors is transiently transfected into the cell. In another specific embodiment, at least one of the vectors is stably transfected into the cell. In an additional embodiment, when expression of the reporter polynucleotide is upregulated, the modulator is an agonist. In an additional

embodiment, when expression of the reporter polynucleotide is downregulated, the modulator is an antagonist. In a further specific embodiment, when the expression of the reporter polynucleotide is downregulated, the modulator is an antagonist. In a specific embodiment, the cell is a mammalian cell. In a further specific embodiment, the mammalian cell is selected from the group consisting of CHO, HepG2, HeLa, COS-1, MCF-7, MDA-MB-231, T47D, ZR-75, MDA-MB-435, BT-20, MDA-MB-468, and HEC-1. In an additional specific embodiment, the estrogen-responsive regulatory element is selected from the group consisting of SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42; SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49; SEQ ID NO:22; SEQ ID NO:26, and SEQ ID NO:8. In an additional specific embodiment, the reporter polynucleotide is luciferase, chloramphenicol acetyltransferase, renilla or  $\beta$ -galactosidase. In a specific embodiment, there is a method of treating breast cancer in an individual comprising the step of administering the antagonist to the individual.

[0027] In another object of the present invention, there is a method of identifying a polypeptide which interacts with an estrogen receptor alpha polypeptide comprising a K303R substitution, comprising introducing to a cell, a vector comprising a polynucleotide which encodes a chimeric polypeptide comprising the estrogen receptor alpha K303R polypeptide and a DNA binding domain; introducing to the cell, a vector comprising a polynucleotide which encodes a chimeric polypeptide comprising a candidate polypeptide and a DNA activation domain; and assaying for an interaction between the DNA binding domain and the DNA activation domain, wherein when the interaction occurs, the candidate polypeptide is the polypeptide which interacts with the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the polypeptide which interacts with the estrogen receptor alpha K303R polypeptide is an antagonist of the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the interaction is assayed by assaying for a change in expression of a reporter sequence. In a specific embodiment, the cell is a yeast cell. In another specific embodiment, the cell is a mammalian cell. In a further specific embodiment, the DNA activation domain and the DNA binding domain are from GAL4 or LexA. In an additional specific embodiment, the reporter sequence is selected from the group consisting of  $\beta$ -galactosidase, luciferase, chloramphenicol acetyltransferase, and renilla. In a specific embodiment, there is a method of treating an individual for breast cancer, comprising administering the antagonist to the individual.



[0028] In another object of the present invention, there is a method of identifying a peptide which interacts with an estrogen receptor alpha K303R polypeptide, comprising obtaining an estrogen receptor alpha K303R polypeptide having an affinity tag and a label; introducing the polypeptide to a substrate comprising a plurality of bacteriophage, wherein the bacteriophage produce a candidate peptide; and determining binding of the polypeptide with the candidate peptide, wherein when the polypeptide binds the candidate peptide, the candidate peptide is the interacting peptide. In a specific embodiment, the label is a color label, a fluorescence label, or a radioactive label. In another specific embodiment, the affinity tag is biotin, GST, histidine, myc, or calmodulin-binding protein.

[0029] In an additional object of the present invention, there is a method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the activity; and determining whether the compound has the activity. In a specific embodiment, the compound having the activity is an antagonist of the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the method further comprises dispersing the compound in a pharmaceutical carrier; and administering a therapeutically effective amount of the compound in the carrier to an individual having the breast cancer.

[0030] Another object of the present invention is the compound obtained by the method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the activity; and determining whether the compound has the activity.

[0031] An additional object of the present invention is a pharmacologically acceptable composition comprising the compound obtained by the method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the activity; and determining whether the compound has the activity; and a pharmaceutical carrier.

[0032] Other and further objects, features, and advantages would be apparent and eventually more readily understood by reading the following specification and be reference to the accompanying drawings forming a part thereof, or any examples of the presently preferred embodiments of the invention given for the purpose of the disclosure.

## BRIEF DESCRIPTION OF THE FIGURES

[0033] FIG. 1 illustrates examples of typical estrogen receptor (ER) expression in premalignant breast lesions as assayed by immunohistochemistry (small dark nuclei are ER-positive cells).

[0034] FIG. 2 illustrates sequence analysis of ER Variant (VAR) and Wild-Type (WT) cDNAs isolated from frozen breast hyperplastic tissue. A portion of the sequencing products are shown for wild-type and variant clones demarcating the location of the G transition and Arg substitution. ER domains A through E and the exons across these domains are shown on the bottom panel with the location of the Lys to Arg change demarcated with a box across exon 4 at the end of domain D.

[0035] FIG. 3 demonstrates detection of the ER VAR sequence in archival breast specimens by identification of WT and VAR ER sequences in one patient with typical hyperplasia (TH). Normal adjacent breast epithelium (N Adj.), TH, and distant normal epithelium (N Dis.) were all available for analysis from this patient. The position of the A908G sequence is indicated by arrows.

[0036] FIG. 4 illustrates growth curves of stable MCF-7 transfectants in response to increasing concentrations of estradiol in the media. Cells were plated at a density of  $2 \times 10^4$  in media containing 10% charcoal-stripped, estrogen-free fetal calf serum and were either left untreated [■] or treated with the indicated estradiol concentrations ( $1 \times 10^{-12}$  [●],  $1 \times 10^{-11}$  [π],  $1 \times 10^{-9}$  [◆] M). The medium was replaced every 48 h and the cells were harvested and counted on days 2, 4, 6, and 8, respectively. Cell number  $\times 10^4$  is shown. Panel A demonstrates untransfected parental MCF-7 cells. Panel B demonstrates vector-alone stably transfected cells. Panels C and D demonstrate cells stably transfected with WT ER. Panels E, F, and D demonstrate cells stably transfected with the mutant ER.

[0037] FIG. 5 demonstrates interaction of the WT and mutant ERs with SRC-1, SRC-2 and SRC-3 *in vitro*.

[0038] FIG. 6 demonstrates detection of the ER Mutant (Mut) in archival breast specimens, including identification of WT and Mut ER alleles in 10 typical breast hyperplasias. Both Mut and WT plasmid DNAs were included as positive controls for the location of the migration of their respective alleles (first two lanes). The ten hyperplastic lesions are labeled 1 through 10.

[0039] FIG. 7 illustrates oligonucleotide mismatch hybridization of one patient with concurrent breast lesions. Laser capture microdissection was used to precisely

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microdissect with an enrichment of >90% cellularity. PCR-amplified fragments were obtained from normal breast epithelium adjacent to a hyperplasia (AB), normal breast epithelium distant from malignant breast lesions (DB), TH, normal skin (NS) and two different DCIS lesions (DCIS 1 and 2) and slotted in duplicate onto nylon membranes (Micro Separation, Inc., Westboro, MA). The panel on the left was hybridized with an oligonucleotide to the WT ER sequence, while the panel on the right was hybridized with an oligonucleotide specific for the Mut sequence.

[0040] FIG. 8A through 8D demonstrates ductal hyperplasias in K303R transgenic mice. FIG. 8E-8F show nontransgenic mammary gland controls.

[0041] FIG. 9 shows a comparison of ductal epithelium from K303R transgenic mice versus nontransgenic mice.

## DETAILED DESCRIPTION OF THE INVENTION

[0042] It will be readily apparent to one skilled in the art that various embodiments and modifications may be made in the invention disclosed herein without departing from the scope and spirit of the invention.

[0043] As used in the specification, "a" or "an" may mean one or more. As used in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" may mean at least a second or more.

### I. Definitions

[0044] The term "A908G mutation" as used herein is defined as an adenine (A) - to- guanine (G) base pair transition at nucleotide position 908 in an estrogen receptor alpha nucleic acid sequence, relative to the first nucleotide of the first codon of the translated amino acid sequence. A skilled artisan recognizes that multiple estrogen receptor alpha nucleic acid sequences exist which are, for example, alternative splice variants. Thus, there are some estrogen receptor alpha nucleic acid sequences of different sizes, and the A908G mutation which is present at nucleotide (nt) 908 in the full-length mutated sequence may no longer be at position 908 in a variant sequence. However, a skilled artisan can readily identify the equivalent or analogous sequence in these variants by sequence homology and comparison, and/or by analyzing locations, arrangements or relationships of splicing manipulations. Thus, an estrogen receptor alpha nucleic acid sequence which contains the indicated mutation yet is

a variant, such as an alternatively spliced form of the sequence, is still within the scope of the present invention.

[0045] The term “agonist” as used herein is defined as a compound or composition which promotes, facilitates, allows, induces, or otherwise assists, activates or increases the function of the estrogen receptor alpha K303R polypeptide.

[0046] The term “antagonist” as used herein is defined as a compound or composition which inhibits, stops, deters, impedes, delays, or otherwise prevents the activity and functioning of the estrogen receptor alpha K303R polypeptide.

[0047] The term “biopsy” as used herein is defined as removal of a tissue from a breast for the purpose of examination, such as to establish diagnosis. Examples of types of biopsies include by application of suction, such as through a needle attached to a syringe; by instrumental removal of a fragment of tissue; by removal with appropriate instruments through an endoscope; by surgical excision, such as of the whole lesion; and the like.

[0048] The term “breast cancer” as used herein is defined as cancer which originates in the breast. In a specific embodiment, the breast cancer spreads to other organs, such as lymph nodes. In a specific embodiment, the breast cancer is invasive and may be metastatic.

[0049] The term “cancer” as used herein is defined as a new growth of tissue comprising uncontrolled and progressive multiplication. In a specific embodiment, upon a natural course the cancer is fatal. In specific embodiments, the cancer is invasive, metastatic, and/or anaplastic (loss of differentiation and of orientation to one another and to their axial framework).

[0050] The term “invasive” as used herein refers to cells which have the ability to infiltrate surrounding tissue. In a specific embodiment, the infiltration results in destruction of the surrounding tissue. In another specific embodiment, the cells are cancer cells. In a preferred embodiment, the cells are breast cancer cells, and the cancer spreads out of a duct into surrounding breast epithelium. In a specific embodiment, “metastatic” breast cancer is within the scope of “invasive.”

[0051] The term “K303R substitution” as used herein is defined as the amino acid substitution which results from the A908G mutation in estrogen receptor alpha nucleic acid sequence. The term “Lys303Arg substitution” is used herein interchangeably. A skilled artisan recognizes that multiple estrogen receptor alpha amino acid sequences exist which are, for example, alternative splice variants. Thus, there are some estrogen receptor alpha amino acid sequences of different sizes, and the K303R substitution which is present in the

full-length mutated sequence may no longer be at position 303 in the variant sequence. However, a skilled artisan can readily identify the equivalent or analogous sequence in these variants by sequence homology and comparison, and/or by analyzing locations, arrangements or relationships of splicing manipulations. Thus, an estrogen receptor alpha amino acid sequence which contains the indicated mutation yet is a variant, such as an alternatively spliced form of the sequence, is still within the scope of the present invention.

**[0052]** The term “laser capture microdissection” as used herein is defined as the use of an infrared (IR) laser beam to remove a desired cell from a undesired cell. In preferred embodiments, the desired cell is a cancer cell and the undesired cell is a normal cell. In another preferred embodiment, the cancer cell is a breast cancer cell.

**[0053]** The term “manual manipulation” as used herein is defined as the selective removal of a desired cell or cells from a undesired cell or cells by hand. In preferred embodiments, the desired cell is a cancer cell and the undesired cell is a normal cell. In another preferred embodiment, the cancer cell is a breast cancer cell.

**[0054]** The term “metastatic” as used herein is defined as the transfer of cancer cells from one organ or part to another not directly connected with it. In a specific embodiment, breast cancer cells spread to another organ or body part, such as lymph nodes.

**[0055]** The term “pre-malignant lesion” as used herein is defined as a collection of cells in a breast with histopathological characteristics which suggest at least one of the cells has an increased risk of becoming breast cancer. A skilled artisan recognizes that the most important pre-malignant lesions recognized today include unfolded lobules (UL; other names: blunt duct adenosis, columnar alteration of lobules), usual ductal hyperplasia (UDH; other names: proliferative disease without atypia, epitheliosis, papillomatosis, benign proliferative disease), atypical ductal hyperplasia (ADH), atypical lobular hyperplasia (ALH), ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (LCIS). Other lesions which may have pre-malignant potential include intraductal papillomas, sclerosing adenosis, and fibroadenomas (especially atypical fibroadenomas). In a specific embodiment, the collection of cells is a lump, tumor, mass, bump, bulge, swelling, and the like. Other terms in the art which are interchangeable with “pre-malignant lesion” include pre-malignant hyperplasia, pre-malignant neoplasia, and the like.

**[0056]** The term “sample from a breast” as used herein is defined as a specimen from any part or tissue of a breast. A skilled artisan recognizes that the sample may be obtained by any method, such as biopsy. In a specific embodiment the sample is obtained by nipple aspirate (see, for example, Sauter *et al.* (1997)). In another specific embodiment, the

sample is from hyperplastic or malignant breast epithelium. In a specific embodiment, the sample is from the epithelium. In another specific embodiment, the sample is from a premalignant lesion. A skilled artisan recognizes that within the scope of the present invention is the embodiment wherein a normal, or benign, sample, such as from an epithelium, is obtained for risk screening.

## II. The Present Invention

[0057] The best current model of breast cancer evolution suggests that most cancers arise from certain premalignant lesions. The present invention is directed to a common (34%) somatic mutation in the estrogen receptor (ER)  $\alpha$  gene in a series of 59 typical hyperplasias, a type of early premalignant breast lesion. The mutation, which affects the border of the hinge and hormone binding domains of ER $\alpha$ , showed increased sensitivity to estrogen as compared to wild-type ER $\alpha$  in stably transfected breast cancer cells, including markedly increased proliferation at subphysiologic levels of estrogen. The mutated ER $\alpha$  exhibits significantly enhanced binding to the TIF-2 (SRC-2) and SRC-3 co-activators and moderately enhanced binding to SRC-1 at low levels of hormone, which in a specific embodiment explains its increased estrogen responsiveness. In a preferred embodiment, this mutation promotes or accelerates the development of cancer from premalignant breast lesions. As such, it is a useful tool for the diagnosis of breast cancer and determination of susceptibility to the development of breast cancer, including determination of the propensity for invasiveness.

[0058] A skilled artisan recognizes the existence of a variety of inherited, or somatically acquired, variations in the DNA of the estrogen receptor alpha gene in cells in a breast sample, which, in the latter case, may differ in a mixture of normal and neoplastic cells. As demonstrated in the Examples herein, those cells having DNA that contain an A908G mutation in the estrogen receptor alpha nucleic acid sequence are or will become cancerous, and particularly will be a cell of a breast cancer which will become metastatic. The present invention is directed to methods and compositions related to detection of the A908G mutation.

[0059] In an embodiment of the present invention there is an isolated estrogen receptor alpha nucleic acid sequence comprising an A908G mutation.

[0060] In another embodiment of the present invention there is an isolated estrogen receptor alpha amino acid sequence comprising a K303R substitution.

[0061] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual, wherein the sample comprises a cell having an estrogen receptor alpha nucleic acid sequence; and assaying the nucleic acid sequence for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer. In a specific embodiment, the sample is from a premalignant lesion of the breast.

[0062] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of invasive breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; and assaying an estrogen receptor alpha nucleic acid sequence from a cell of the sample for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence detects susceptibility of the premalignant lesion to develop into the invasive breast cancer. In a specific embodiment, the sample is from a premalignant lesion of the breast.

[0063] In an additional embodiment of the present invention there is a method of detecting susceptibility to development of invasive breast cancer from a premalignant lesion in a breast, comprising the steps of obtaining a sample from the premalignant lesion; dissecting the sample to differentiate hyperplastic cells in the sample from nonhyperplastic cells; and assaying an estrogen receptor alpha nucleic acid sequence from the hyperplastic cell of the sample for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence detects susceptibility of the premalignant lesion to develop into the invasive breast cancer. In a specific embodiment, the dissection step comprises removal of the hyperplastic cells from the sample by manual manipulation or by laser capture microdissection. In another specific embodiment, the sample is obtained by biopsy. In a specific embodiment, the assaying step comprises sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection, or a combination thereof. In an additional specific embodiment, the assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or is by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence. In a further specific embodiment, the assaying step is by detection of SNPs by methods well known in the art.

[0064] In an additional embodiment of the present invention there is a method of classifying breast cancer in an individual, comprising the steps of obtaining from the individual a sample from the breast, wherein the sample contains a cancer cell; and assaying

an estrogen receptor alpha nucleic acid sequence from the cell of the sample for an A908G mutation, wherein the presence of the mutation identifies the breast cancer to be invasive breast cancer. In a specific embodiment, the sample is obtained by biopsy. In another specific embodiment, the assaying step is selected from the group consisting of sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection, and a combination thereof.

[0065] A skilled artisan recognizes that there are a variety of methods to detect a mutation in a nucleic acid sequence in addition to these methods. Methods regarding allele-specific probes for analyzing particular nucleotide sequences are described by *e.g.*, Saiki *et al.*, Nature 324, 163-166 (1986); Dattagupta, EP 235,726 (U.S. 836,378 (03/05/86); U.S. 943,006 (12/29/86)); Saiki, WO 89/11548 (U.S. 197,000 (05/20/88); U.S. 347,495 (05/04/89)). Allele-specific probes are typically used in pairs. One member of the pair shows perfect complementarity to a wildtype allele and the other members to a variant allele. In idealized hybridization conditions to a homozygous target, such a pair shows an essentially binary response. That is, one member of the pair hybridizes and the other does not. An allele-specific primer hybridizes to a site on target DNA overlapping the particular site in question and primes amplification of an allelic form to which the primer exhibits perfect complementarity (Gibbs, 1989). This primer is used in conjunction with a second primer which hybridizes at a distal site. Amplification proceeds from the two primers leading to a detectable product signifying the particular allelic form is present. A control is usually performed with a second pair of primers, one of which shows a single base mismatch at the polymorphic site and the other of which exhibits perfect complementarity to a distal site. The single-base mismatch impairs amplification and little, if any, amplification product is generated.

[0066] Particular nucleic acid sites can also be identified by hybridization to oligonucleotide arrays. An example is described in WO 95/11995, which includes arrays having four probe sets. A first probe set includes overlapping probes spanning a region of interest in a reference sequence. Each probe in the first probe set has an interrogation position that corresponds to a nucleotide in the reference sequence. That is, the interrogation position is aligned with the corresponding nucleotide in the reference sequence when the probe and reference sequence are aligned to maximize complementarity between the two. For each probe in the first set, there are three corresponding probes from three additional probe sets. Thus, there are four probes corresponding to each nucleotide in the reference sequence. The probes from the three additional probe sets are identical to the corresponding probe from the



first probe set except at the interrogation position, which occurs in the same position in each of the four corresponding probes from the four probe sets, and is occupied by a different nucleotide in the four probe sets. Such an array is hybridized to a labeled target sequence, which may be the same as the reference sequence, or a variant thereof. The identity of any nucleotide of interest in the target sequence can be determined by comparing the hybridization intensities of the four probes having interrogation positions aligned with that nucleotide. The nucleotide in the target sequence is the complement of the nucleotide occupying the interrogation position of the probe with the highest hybridization intensity.

[0067] WO 95/11995 also describes subarrays that are optimized for detection of variant forms of a precharacterized nucleotide site. A subarray contains probes designed to be complementary to a second reference sequence, which can be an allelic variant of the first reference sequence. The second group of probes is designed by the same principles as above except that the probes exhibit complementarity to the second reference sequence. The inclusion of a second group can be particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur within a short distance commensurate with the length of the probes (*i.e.*, two or more mutations within 9 to 21 bases).

[0068] An additional strategy for detecting a particular nucleotide site uses an array of probes is described in EP 717,113 (U.S. 327,525 (10/21/94)). In this strategy, an array contains overlapping probes spanning a region of interest in a reference sequence. The array is hybridized to a labeled target sequence, which may be the same as the reference sequence or a variant thereof. If the target sequence is a variant of the reference sequence, probes overlapping the site of variation show reduced hybridization intensity relative to other probes in the array. In arrays in which the probes are arranged in an ordered fashion stepping through the reference sequence (*e.g.*, each successive probe has one fewer 5' base and one more 3' base than its predecessor), the loss of hybridization intensity is manifested as a "footprint" of probes approximately centered about the point of variation between the target sequence and reference sequence.

[0069] Mundy, C. R. (U.S. Pat. No. 4,656,127), for example, discusses a method for determining the identity of the nucleotide present at a particular site that employs a specialized exonuclease-resistant nucleotide derivative. A primer complementary to the allelic sequence immediately 3' to the site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative

present, then that derivative will be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the site of the target molecule was complementary to that of the nucleotide derivative used in the reaction. The Mundy method has the advantage that it does not require the determination of large amounts of extraneous sequence data. It has the disadvantages of destroying the amplified target sequences, and unmodified primer and of being extremely sensitive to the rate of polymerase incorporation of the specific exonuclease-resistant nucleotide being used.

[0070] Cohen, D. *et al.* (French Patent 2,650,840 (U.S. 4,420,902 (12/20/83)); PCT Appln. No. WO91/02087) discuss a solution-based method for determining the identity of the nucleotide of a particular site. As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to allelic sequences immediately 3' to the site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the site will become incorporated onto the terminus of the primer.

[0071] An alternative method, known as Genetic Bit Analysis or GBA™ is described by Goelet, P. *et al.* (PCT Appln. No. 92/15712 (U.S. 664,837 (03/05/91); U.S. 775,786 (10/11/91) ). The method of Goelet, P. *et al.* uses mixtures of labeled terminators and a primer that is complementary to the sequence 3' to a site in question. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the site of the target molecule being evaluated. In contrast to the method of Cohen *et al.* (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. *et al.* is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase. It is thus easier to perform, and more accurate than the method discussed by Cohen.

[0072] An alternative approach, the "Oligonucleotide Ligation Assay" ("OLA") (Landegren, U. *et al.*, Science 241:1077-1080 (1988)) has also been described as capable of detecting a nucleotide sequence variation. The OLA protocol uses two oligonucleotides which are designed to be capable of hybridizing to abutting sequences of a single strand of a target. One of the oligonucleotides is biotinylated, and the other is detectably labeled. If the precise complementary sequence is found in a target molecule, the oligonucleotides will hybridize such that their termini abut, and create a ligation substrate. Ligation then permits

the labeled oligonucleotide to be recovered using avidin, or another biotin ligand. Nickerson, D. A. *et al.* have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson, D. A. *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 87:8923-8927 (1990). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA. In addition to requiring multiple, and separate, processing steps, one problem associated with such combinations is that they inherit all of the problems associated with PCR and OLA.

[0073] Recently, several primer-guided nucleotide incorporation procedures for assaying particular sites in DNA have been described (Komher, J. S. *et al.*, Nucl. Acids. Res. 17:7779-7784 (1989); Sokolov, B. P., Nucl. Acids Res. 18:3671 (1990); Syv anen, A. -C., *et al.*, Genomics 8:684-692 (1990); Kuppuswamy, M. N. *et al.*, Proc. Natl. Acad. Sci. (U.S.A.) 88:1143-1147 (1991); Prezant, T. R. *et al.*, Hum. Mutat. 1:159-164 (1992); Ugozzoli, L. *et al.*, GATA 9:107-112 (1992); Nyren, P. *et al.*, Anal. Biochem. 208:171-175 (1993)).

[0074] In an additional specific embodiment of the present invention an assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence.

[0075] In another embodiment of the present invention there is a method of diagnosing breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual, wherein the sample comprises a cell having an estrogen receptor alpha nucleic acid sequence; and assaying the nucleic acid sequence for an A908G mutation, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer.

[0076] In another embodiment of the present invention there is a method of diagnosing breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; dissecting the sample to differentiate a cell suspected of being cancerous from a noncancerous cell; and assaying the cell suspected of being cancerous for an A908G mutation in an estrogen receptor alpha nucleic acid sequence, wherein the presence of the mutation in the nucleic acid sequence indicates the individual has breast cancer. In a specific embodiment, the dissection step comprises removal of the cells suspected of being cancerous from the sample by manual manipulation or by laser capture microdissection. In a specific embodiment, the sample is obtained by biopsy. In another specific embodiment, the assaying step is selected from the group consisting of sequencing, single stranded conformation polymorphism, mismatch oligonucleotide mutation detection,

and a combination thereof. In an additional specific embodiment, the assaying step is by antibody detection with antibodies to the A908G mutation of the estrogen receptor alpha nucleic acid sequence or is by antibody detection with antibodies to an acetylated estrogen receptor alpha amino acid sequence. In a specific embodiment, the mutation is detected by SNP analysis, using standard methods in the art. Some methods use extendable primers for incorporating radiolabeled nucleotides, which can then be detected by fluorescence or resonance. For example, PerkinElmer<sup>TM</sup> (Shelton, CT) has the AcycloPrime<sup>TM</sup> fluorescence polarization SNP detection system which utilizes terminator labeled nucleotides to facilitate detection of the SNP upon fluorescence polarization. Also, Applied Biosystems (Foster City, CA) has the ABI PRISM<sup>®</sup> turbo TaqMan<sup>®</sup> probes for genotyping by allelic detection which utilizes fluorescent dyes, such as VIC<sup>TM</sup>, and TET and 6-FAM, for detection. In a specific embodiment, the thymidine residues of the probes are replaced with 5-propyne-2'-deoxyuridine, which increases the T<sub>m</sub> of these probes by approximately 1 °C per substitution and facilitates design of a shorter probe for greater accuracy.

[0077] In another embodiment of the present invention there is a kit for diagnosing an A908G mutation in an estrogen receptor alpha nucleic acid sequence, comprising at least one primer selected from the group consisting of SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:33, SEQ ID NO:34, and SEQ ID NO:35. In a specific embodiment, the kit contains primers which are extendable. In an alternative specific embodiment, the kit contains primers which are nonextendable.

[0078] In another embodiment of the present invention there is a monoclonal antibody that binds immunologically to an acetylated estrogen receptor alpha amino acid sequence, or an antigenic fragment thereof.

[0079] In another embodiment of the present invention there is a monoclonal antibody that binds immunologically to an A908G mutation in an estrogen receptor alpha nucleic acid sequence.

[0080] In an additional embodiment of the present invention there is a method to correct a G mutation at nucleotide 908 of an estrogen receptor alpha nucleic acid sequence in a cell of an individual, comprising the step of administering to the cell an estrogen receptor alpha nucleic acid sequence comprising an A at nucleotide 908. In a specific embodiment, the estrogen receptor alpha nucleic acid sequence comprising an A at nucleotide 908 is present on a vector. In another specific embodiment, the vector is selected from the group consisting of plasmid, viral vector, liposome, and a combination thereof. In an additional

specific embodiment, the viral vector is selected from the group consisting of adenoviral vector, retroviral vector, adeno-associated viral vector, or a combination thereof.

[0081] In an additional embodiment of the present invention there is a method to prevent breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; identifying in the sample an A908G mutation in a nucleic acid sequence of estrogen receptor alpha; and correcting the A908G mutation, wherein the correction results in the prevention of the breast cancer. In a specific embodiment, the breast sample is from a premalignant lesion of the breast. In another specific embodiment, the correction step comprises administering an estrogen receptor alpha nucleic acid sequence comprising a G at nucleotide 908 to a cell comprising an estrogen receptor alpha nucleic acid sequence containing the A908G mutation.

[0082] In an additional embodiment of the present invention there is a method to treat breast cancer in an individual, wherein an estrogen receptor alpha nucleic acid sequence in a breast cell of the individual has an A908G mutation, comprising the step of administering to the cell an estrogen receptor alpha nucleic acid sequence comprising a G at nucleotide 908.

[0083] In another embodiment of the present invention there is a method to prevent breast cancer in an individual, comprising the steps of obtaining a sample from a breast of the individual; identifying in the sample an arginine at amino acid residue 303 in an amino acid sequence of estrogen receptor alpha; and administering to the individual an amino acid sequence of estrogen receptor alpha comprising a lysine at amino acid residue 303, wherein the administration results in the prevention of the breast cancer. In a specific embodiment, the breast sample is from a premalignant lesion of the breast.

[0084] In an object of the present invention there is a method of identifying a modulator of an estrogen receptor alpha K303R polypeptide, comprising providing a candidate modulator; admixing the candidate modulator with an isolated compound or cell, or a suitable experimental animal; measuring one or more characteristics of the compound, cell or animal; and comparing the characteristic measured with the characteristic of the compound, cell or animal in the absence of the candidate modulator, wherein a difference between the measured characteristics indicates that the candidate modulator is the modulator of the compound, cell or animal.

[0085] In another object of the present invention, there is a method of screening for a modulator of an estrogen receptor alpha polypeptide comprising a K303R substitution, comprising introducing to a cell a vector comprising a nucleic acid sequence which encodes the estrogen receptor alpha K303R polypeptide; a vector comprising at least one estrogen-

responsive regulatory element operatively linked to a reporter polynucleotide; and a test agent; and assaying expression of the reporter polynucleotide in the presence of the test agent, wherein the test agent is the modulator when the reporter polynucleotide expression changes in the presence of the test agent. In a specific embodiment, at least one of the vectors is transiently transfected into the cell. In another specific embodiment, at least one of the vectors is stably transfected into the cell. In an additional embodiment, when expression of the reporter polynucleotide is upregulated, the modulator is an agonist. In an additional embodiment, when expression of the reporter polynucleotide is downregulated, the modulator is an antagonist. In a further specific embodiment, when the expression of the reporter polynucleotide is downregulated, the modulator is an antagonist. In a specific embodiment, the cell is a mammalian cell. In a further specific embodiment, the mammalian cell is selected from the group consisting of CHO, HepG2, HeLa, COS-1, MCF-7, MDA-MB-231, T47D, ZR-75, MDA-MB-435, BT-20, MDA-MB-468, and HEC-1. In an additional specific embodiment, the estrogen-responsive regulatory element is selected from the group consisting of SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42; SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49; SEQ ID NO:22; SEQ ID NO:26, and SEQ ID NO:8. In an additional specific embodiment, the reporter polynucleotide is luciferase, chloramphenicol acetyltransferase, renilla or  $\beta$ -galactosidase. In a specific embodiment, there is a method of treating breast cancer in an individual comprising the step of administering the antagonist to the individual.

[0086] In another object of the present invention, there is a method of identifying a polypeptide which interacts with an estrogen receptor alpha polypeptide comprising a K303R substitution, comprising introducing to a cell, a vector comprising a polynucleotide which encodes a chimeric polypeptide comprising the estrogen receptor alpha K303R polypeptide and a DNA binding domain; introducing to the cell, a vector comprising a polynucleotide which encodes a chimeric polypeptide comprising a candidate polypeptide and a DNA activation domain; and assaying for an interaction between the DNA binding domain and the DNA activation domain, wherein when the interaction occurs, the candidate polypeptide is the polypeptide which interacts with the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the polypeptide which interacts with the estrogen receptor alpha K303R polypeptide is an antagonist of the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the interaction is assayed by assaying for a change in

expression of a reporter sequence. In a specific embodiment, the cell is a yeast cell. In another specific embodiment, the cell is a mammalian cell. In a further specific embodiment, the DNA activation domain and the DNA binding domain are from GAL4 or LexA. In an additional specific embodiment, the reporter sequence is selected from the group consisting of  $\beta$ -galactosidase, luciferase, chloramphenicol acetyltransferase, and renilla. In a specific embodiment, there is a method of treating an individual for breast cancer, comprising administering the antagonist to the individual.

[0087] In another object of the present invention, there is a method of identifying a peptide which interacts with an estrogen receptor alpha K303R polypeptide, comprising obtaining an estrogen receptor alpha K303R polypeptide having an affinity tag and a label; introducing the polypeptide to a substrate comprising a plurality of bacteriophage, wherein the bacteriophage produce a candidate peptide; and determining binding of the polypeptide with the candidate peptide, wherein when the polypeptide binds the candidate peptide, the candidate peptide is the interacting peptide. In a specific embodiment, the label is a color label, a fluorescence label, or a radioactive label. In another specific embodiment, the affinity tag is biotin, GST, histidine, myc, or calmodulin-binding protein.

[0088] In an additional object of the present invention, there is a method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the activity; and determining whether the compound has the activity. In a specific embodiment, the compound having the activity is an antagonist of the estrogen receptor alpha K303R polypeptide. In a specific embodiment, the method further comprises dispersing the compound in a pharmaceutical carrier; and administering a therapeutically effective amount of the compound in the carrier to an individual having the breast cancer.

[0089] Another object of the present invention is the compound obtained by the method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the activity; and determining whether the compound has the activity.

[0090] An additional object of the present invention is a pharmacologically acceptable composition comprising the compound obtained by the method of identifying a compound for the treatment of breast cancer associated with an estrogen receptor alpha K303R polypeptide, comprising the steps of obtaining a compound suspected of having the

activity; and determining whether the compound has the activity; and a pharmaceutical carrier.

### III. Estrogen Receptor Alpha

[0091] Estrogen, mediated through the estrogen receptor (ER), plays a major role in regulating the growth and differentiation of normal breast epithelium (Pike *et al.*, 1993; Henderson *et al.*, 1988). It stimulates cell proliferation and regulates the expression of other genes, including the progesterone receptor (PgR). PgR then mediates the mitogenic effect of progesterone, further stimulating proliferation (Pike *et al.*, 1993; Henderson *et al.*, 1988). Several studies have assessed ER expression in normal breast epithelium and premalignant lesions. Studies of normal terminal duct lobular units (TDLUs) reported that nearly all (over 90%) express ER, but in a minority (averaging about 30%) of cells for all ages combined (Schmitt, 1995; Mohsin *et al.*, 2000; Allegra *et al.*, 1979; Peterson *et al.*, 1986; Ricketts *et al.*, 1991). In premenopausal women, the average proportion of ER-positive cells in TDLUs is somewhat lower (about 20%), and varies with the menstrual cycle, being twice as high during the follicular as the luteal phase (Ricketts *et al.*, 1991). Proliferation in TDLUs peaks during the luteal phase (Potten *et al.*, 1988), suggesting that the normal mitogenic effect of estrogen may be partially delayed or indirect and mediated by downstream interactions such as that between progesterone and PgR. In postmenopausal women, the average proportion of ER-positive cells in TDLUs is relatively high (about 50%) and stable in the absence of hormone replacement therapy (Mohsin *et al.*, 2000). Very little is known about ER expression in ULs, although one preliminary study reported that virtually all expressed the receptor in over 90% of cells (Mohsin *et al.*, 2000). A few studies have evaluated ER in ADH and collectively agreed that nearly all lesions express very high levels in nearly all cells (Schmitt, 1995; Mohsin *et al.*, 2000; Barnes and Masood, 1990). Many studies have evaluated ER in DCIS and, on average, about 75% of all cases expressed the receptor (Mohsin *et al.*, 2000; Zafrani *et al.*, 1994; Albonico *et al.*, 1996; Berardo *et al.*, 1996; Barnes and Masood, 1990; Helin *et al.*, 1989; Giri *et al.*, 1989; Chaudhuri *et al.*, 1993; Poller *et al.*, 1993; Pallis *et al.*, 1992; Leal *et al.*, 1995; Karayiannakis *et al.*, 1996; Bose *et al.*, 1996). Expression varied with histological differentiation, being highest in non-comedo (non-mammary ductal) lesions, where up to 100% showed expression in over 90% of cells, and lowest in comedo lesions, where only about 30% showed expression in a minority of cells. ER was not expressed in about 25% of DCIS and these were predominately high-grade comedo lesions. Over 90% of LCIS expressed high levels of ER in nearly all cells (Fisher *et al.*, 1996; Rudas *et al.*, 1997;



Querzoli *et al.*, 1998; Libby *et al.*, 1998; Giri *et al.*, 1989; Pallis *et al.*, 1992; Paertschuk *et al.*, 1990), which is similar in ALH in a specific embodiment.

[0092] Prolonged estrogen exposure is an important risk factor for developing IBC, perhaps by allowing random genetic alterations to accumulate in normal cells stimulated to proliferate (Henderson *et al.* 1988), which may also be true for cells in premalignant lesions. The very high levels of ER observed in nearly all premalignant lesions (FIG. 1) may contribute to their increased proliferation relative to normal cells by allowing them to respond more effectively to any level of estrogen, even the low concentrations seen in postmenopausal women (Mohsin *et al.*, 2000). FIG. 1 illustrates examples of typical estrogen receptor expression in premalignant breast lesions as assessed by immunohistochemistry (small dark nuclei are ER-positive cells). Terminal duct lobular units (TDLUs) in premenopausal (pre) women usually contain relatively few ER positive cells. In contrast, the majority of cells in TDLUs of postmenopausal (post) express ER. Most premalignant breast lesions show very high levels of ER in nearly all cells, including unfolded lobules (ULs), atypical ductal hyperplasias (ADHs), low grade “non-comedo” ductal carcinoma in situ (ncDCIS), atypical lobular hyperplasias (ALHs), and lobular carcinoma in situ (LCIS). The only significant exception is high grade “comedo” DCIS (cDCIS), which often show low or no ER expression.

[0093] In addition to increased levels of expression, there may be other alterations of ER resulting in increased growth in premalignant lesions. For example, in one recent study (Mohsin *et al.*, 2000), proliferation was measured in TDLUs and premalignant lesions from the same breasts in a large number of patients stratified by menopausal status. Proliferation rates in TDLUs were nearly 3-fold lower in postmenopausal compared to premenopausal women, consistent with the expected mitogenic effect of estrogen and progesterone in normal cells. In contrast, the difference in proliferation in premalignant lesions stratified by menopausal status was less than half that of normal cells, suggesting that the hormonal regulation of proliferation in these lesions, in a specific embodiment, is fundamentally abnormal. It is an object of the present invention to diagnose such an abnormality by identifying an A908G mutation in estrogen receptor alpha nucleic acid sequence or a K303R substitution in the amino acid sequence.

#### IV. Premalignant Lesions of the Breast

[0094] Premalignant lesions of the breast are very common, and they are being diagnosed more frequently due to increasing public awareness and screening mammography. They are currently defined by their histological features and their prognosis is imprecisely estimated based on indirect epidemiological evidence (Page and Dupont, 1993). While lesions within specific categories look alike histologically, there must be underlying biological differences causing a subset to progress to IBC. Studies identifying biological prognostic factors in premalignant disease are beginning to emerge (see discussions in Page and Jensen, 1994; Page, 1995; Page *et al.*, 1998; Lakhani, 1999). The histopathological characteristics and anatomic markers associated with premalignant lesions are well known in the art (Cardiff *et al.*, 1977; Bocker, 1997; Page and Dupont, 1990; Stoll, 1999; Lishman and Lakhani, 1999, each of which are incorporated by reference herein in their entirety).

[0095] For example, preliminary results from two recent studies suggest that increased levels of ER in normal breast epithelium (Kahn *et al.*, 1998) and certain premalignant lesions (UL, ADH, DCIS) (Mohsin *et al.*, 2000) may be associated with a slightly elevated (2-to-3-fold) risk of developing IBC, and assessing ER status may eventually be important in clinical management. Its most promising role may be in identifying patients with high-risk premalignant lesions who might benefit from hormonal therapy. In the recent NSABP P-1 chemoprevention clinical trial (Fisher *et al.*, 1998), patients with a history of ADH receiving tamoxifen experienced a dramatic decrease (85%) in breast cancer incidence. Nearly all ADH express very high levels of ER, suggesting that highly ER positive premalignant lesions may be particularly susceptible to hormonal therapy. The success of this trial is proof-of-principle that targeting biological alterations in premalignant disease is a rational strategy for the chemoprevention of breast cancer.

[0096] Even though microscopic in size, all types of premalignant breast lesions are tumors which expand terminal duct lobular units (TDLUs) and proximal ducts to many times their normal size. Many studies, using a variety of techniques, have measured the magnitude of proliferation in TDLUs and premalignant lesions (Table 1).

Table 1. Growth (proliferation and apoptosis) in premalignant breast lesions.

	TDLU	UL	ADH	DCIS	ALH	LCIS
Average % Proliferation	2%	5%	5%	15%	"low"	2%
Average % Apoptosis	0.6%	"low"	.03	5%	"low"	"low"

## Abbreviations:

TDLUs	=	terminal duct lobular units.
ULs	=	unfolded lobules.
ADH	=	atypical ductal hyperplasia.
DCIS	=	ductal carcinoma in situ.
ALH	=	atypical lobular hyperplasia.
LCIS	=	lobular carcinoma in situ.

[0097] Proliferation in TDLUs averaged only about 2% overall (Meyer, 1977; Ferguson and Anderson, 1981; Joshi *et al.*, 1986; Longacre and Bartow, 1986; Russo *et al.*, 1987; Going *et al.*, 1988; Potten *et al.*, 1988; Kamel *et al.*, 1989; Schmitt, 1995; Visscher *et al.*, 1996; Mohsin *et al.*, 2000). In premenopausal women the rate fluctuates with the menstrual cycle and is two-fold higher in the luteal than the follicular phase (Potten *et al.*, 1988). The association between hormonal status and proliferation emphasizes the importance of estrogen and progesterone as mitogens for normal breast epithelium (Pike *et al.*, 1993). Proliferation has not been evaluated in unfolded lobules (ULs) with the exception of one preliminary study reporting an average rate of about 5%, which is still 2-to-3-fold higher than in normal TDLUs (Mohsin *et al.*, 2000). Studies of ADH also observed rates averaging about 5% (Mohsin *et al.*, 2000; De Potter *et al.*, 1987; Hoshi *et al.*, 1995). Proliferation has been studied more extensively in DCIS than any other type of premalignant lesion (Mohsin *et al.*, 2000; Meyer, 1986; Locker *et al.*, 1990; Poller *et al.*, 1994; Bobrow *et al.*, 1994; Zafrani *et al.*, 1994; Albonico *et al.*, 1996; Berardo *et al.*, 1996). Rates averaged about 5% in histologically low-grade “non-comedo” ductal carcinoma in situ (DCIS) compared to 20% in high-grade “comedo” lesions. The wide-spread practice of dichotomizing DCIS into non-comedo and comedo subtypes is misleading in the sense that, similar to invasive breast cancer (IBC), DCIS shows tremendous histological diversity along a continuum ranging from very well to very poorly differentiated, and grading systems have been developed which more accurately convey this diversity (Berardo *et al.*, 1996). Proliferation is proportional to differentiation along this continuum, with rates averaging as low as 1% in the lowest grade to more than 70% in the highest grade lesions (Bobrow *et al.*, 1994; Berardo *et al.*, 1996). Proliferation has not been formally studied in ALH but is probably similar to LCIS where the reported average is about 2% (Fisher *et al.*, 1996; Rudas *et al.*, 1997; Querzoli *et al.*, 1998; Libby *et al.*, 1998).

[0098] The overall growth of premalignant breast lesions can be viewed simplistically as a balance between cell proliferation and cell death. On average, the cells in all types of premalignant lesions proliferate faster than normal cells in TDLUs, contributing

to their positive growth imbalance. Much less is known about cell death in this setting (Table 1). One preliminary study reported significantly lower rates of apoptosis in atypical ductal hyperplasia (ADH) (0.3%) compared to TDLUs (0.6%) in the same breasts, suggesting that the growth of ADH may be the result of both increased proliferation and decreased cell death compared to normal cells (Prosser *et al.*, 1997). However, a few studies have reported rates of apoptosis in DCIS that are much higher (up to 10-fold) than typically seen in normal cells (Prosser *et al.*, 1997; Bodis *et al.*, 1996; Harn *et al.*, 1997), yet DCIS have a profound positive growth imbalance, suggesting that the relationship between cell proliferation and death may not always be accurately portrayed by the static methods used to measure these dynamic processes. Like proliferation, apoptosis seems to vary with histological differentiation in DCIS, being much lower in non-comedo (averaging 0.7%) than comedo (averaging 5.6%) lesions (Prosser *et al.*, 1997). Disturbances of the equilibrium between cell proliferation and death probably result from alterations of several normal growth-regulating mechanisms, including those involving sex hormones, oncogenes, tumor suppressor genes, and many other genetic and epigenetic abnormalities.

## V. Laser Capture Microdissection

[0099] Developments in gene sequencing and amplification techniques, among others, now allow scientists to extract DNA or RNA from tissue biopsies and cytological smears for pinpoint molecular analysis, such as a point mutation in a nucleic acid sequence. The efficacy of these sophisticated genetic testing methods, however, depends on the purity and precision of the cell populations being analyzed. Simply homogenizing the biopsy sample results in an impure combination of healthy and diseased tissue. Using mechanical tools to manually separate cells of interest from the histologic section is time-consuming and extremely labor-intensive. None of these methods offers the ease, precision and efficiency necessary for modern molecular diagnosis.

[0100] The process of laser capture microdissection (LCM) circumvents many problems in the art regarding accuracy, efficiency and purity. A laser beam focally activates a special transfer film which bonds specifically to cells identified and targeted by microscopy within the tissue section. The transfer film with the bonded cells is then lifted off the thin tissue section, leaving all unwanted cells behind (which would contaminate the molecular purity of subsequent analysis). The transparent transfer film is applied to the surface of the tissue section. Under the microscope, the diagnostic pathologist or researcher views the thin

tissue section through the glass slide on which it is mounted and chooses microscopic clusters of cells to study. When the cells of choice are in the center of the field of view, the operator pushes a button which activates a near IR laser diode integral with the microscope optics. The pulsed laser beam activates a precise spot on the transfer film immediately above the cells of interest. At this precise location the film melts and fuses with the underlying cells of choice. When the film is removed, the chosen cell(s) are tightly held within the focally expanded polymer, while the rest of the tissue is left behind. This allows multiple homogeneous samples within the tissue section or cytological preparation to be targeted and pooled for extraction of molecules and analysis.

**[0101]** In a commercial system, such as with the instruments and methods of Arcturus (Mountain View, CA) (<http://www.arctur.com/>), the film is permanently bonded to the underside of a transparent vial cap. A mechanical arm precisely positions the transfer surface onto the tissue. The microscope focuses the laser beam to discrete sizes (presently either 30 or 60 micron diameters), delivering precise pulsed doses to the targeted film. Targeted cells are transferred to the cap surface, and the cap is placed directly onto a vial for molecular processing. The size of the targeting pulses is selected by the operator. The cells adherent to the film retain their morphologic features, and the operator can verify that the correct cells have been procured.

**[0102]** Examples of LCM with Breast Tissue include those available at [http://www.arctur.com/technology/lcm\\_examples/ex\\_breast.html](http://www.arctur.com/technology/lcm_examples/ex_breast.html).

**[0103]** Methods regarding the specific preparations and techniques associated with LCM are well known in the art and are provided at (<http://www.arctur.com/technology/protocols.html>), including: Paraffin-Embedded Tissue, Frozen Tissue, White Blood Cell Cytospin, De-Paraffinization of Tissue Sections, Hematoxylin and Eosin Staining, Immunohistochemical Staining (IHC), Intercalator Dye Staining (Fluorescence), Methyl Green Staining, Nuclear Fast Red Staining, and Toluidine Blue O Staining.

**[0104]** An example of Laser Capture Microdissection steps, particularly for use with Acturus instruments, includes the following:

**[0105]** 1. Prepare. Follow routine protocols for preparing a tissue or smear on a standard microscope slide. Apply a Prep Strip™ to flatten the tissue and remove loose debris prior to LCM.

**[0106]** 2. Place. Place a CapSure™ HS onto the tissue in the area of interest. The CapSure™ HS is custom designed to keep the transfer film out of contact with the tissue.

[0107] 3. Capture. Pulse the low power infrared laser. The laser activates the transfer film which then expands down into contact with the tissue. The desired cell(s) adhere to the CapSure™ HS transfer film.

[0108] 4. Microdissect. Lift the CapSure™ HS film carrier, with the desired cell(s) attached to the film surface. The surrounding tissue remains intact.

[0109] 5. Extract. Snap the ExtracSure™ onto the CapSure™ HS. The ExtracSure™ is designed to accept low volumes of digestion buffer while sealing out any non-selected material from the captured cells. Pipette the extraction buffer directly into the digestion well of the ExtracSure™. Place a microcentrifuge tube on top.

[0110] 6. Analyze. Invert the microcentrifuge tube. After centrifuging, the lysate will be at the bottom of the tube. The cell contents, DNA, RNA or protein, are ready for subsequent molecular analysis.

## VI. Mismatch Oligonucleotide Mutation Detection

[0111] A skilled artisan recognizes that one method to identify a point mutation in a nucleic acid sequence is by mismatch oligonucleotide mutation detection, also referred to by other names such as oligonucleotide mismatch detection. In a specific embodiment, a nucleic acid sequence comprising the site to be assayed for the mutation is amplified from a sample, such as by polymerase chain reaction, and a mutation is detected with mutation-specific oligonucleotide probe hybridization of Southern or slot blots, or a combination thereof.

[0112] In a specific embodiment of the present invention, an A908G mutation in estrogen receptor alpha nucleic acid sequence is identified by methods and/or kits employing oligonucleotide mismatch detection.

## VII. Single-Strand Conformation Polymorphism

[0113] Single-strand conformation polymorphism (SSCP) (Orita *et al.*, 1989) facilitates detection of polymorphisms, such as single base pair transitions, through mobility shift analysis on a neutral polyacrylamide gel by methods well known in the art. In specific embodiments, the method is subsequent to polymerase chain reaction or restriction enzyme digestion, either of which is followed by denaturation for separation of the strands. The single stranded species are transferred onto a support such as a nylon membrane, and the mobility shift is detected by hybridization with a nick-translated DNA fragment or with

RNA. In alternative embodiments, the single stranded product is itself labeled, such as with radioactivity, for identification. Samples manifesting migration shifts in SSCP gels in a specific embodiment are analyzed further by other well known methods, such as by DNA sequencing.

[0114] In a specific embodiment of the present invention, an A908G mutation in estrogen receptor alpha nucleic acid sequence is identified by methods and/or kits employing single-strand conformation polymorphism.

### VIII. Site-Directed Mutagenesis

[0115] Structure-guided site-specific mutagenesis represents a powerful tool for the dissection and engineering of protein-ligand interactions (Wells, 1996, Braisted *et al.*, 1996). The technique provides for the preparation and testing of sequence variants by introducing one or more nucleotide sequence changes into a selected DNA.

[0116] Site-specific mutagenesis uses specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent, unmodified nucleotides. In this way, a primer sequence is provided with sufficient size and complexity to form a stable duplex on both sides of the deletion junction being traversed. A primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

[0117] The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double-stranded plasmids are also routinely employed in site-directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

[0118] In general, one first obtains a single-stranded vector, or melts two strands of a double-stranded vector, which includes within its sequence a DNA sequence encoding the desired protein or genetic element. An oligonucleotide primer bearing the desired mutated sequence, synthetically prepared, is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions. The hybridized product is subjected to DNA polymerizing enzymes such as *E. coli* polymerase I (Klenow fragment) in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed, wherein one strand encodes the original non-

mutated sequence, and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

[0119] Comprehensive information on the functional significance and information content of a given residue of protein can best be obtained by saturation mutagenesis in which all 19 amino acid substitutions are examined. The shortcoming of this approach is that the logistics of multiresidue saturation mutagenesis are daunting (Warren *et al.*, 1996, Brown *et al.*, 1996; Zeng *et al.*, 1996; Burton and Barbas, 1994; Yelton *et al.*, 1995; Jackson *et al.*, 1995; Short *et al.*, 1995; Wong *et al.*, 1996; Hilton *et al.*, 1996). Hundreds, and possibly even thousands, of site specific mutants must be studied. However, improved techniques make production and rapid screening of mutants much more straightforward. See also, U.S. Patents 5,798,208 and 5,830,650, for a description of “walk-through” mutagenesis.

[0120] Other methods of site-directed mutagenesis are disclosed in U.S. Patents 5,220,007; 5,284,760; 5,354,670; 5,366,878; 5,389,514; 5,635,377; and 5,789,166.

## IX. Nucleic Acid Detection

[0121] In addition to their use in directing the expression of estrogen receptor alpha wildtype or mutant proteins, polypeptides and/or peptides, the nucleic acid sequences disclosed herein have a variety of other uses. For example, they have utility as probes or primers for embodiments involving nucleic acid hybridization.

### A. Hybridization

[0122] The use of a probe or primer of between 13 and 100 nucleotides, preferably between 17 and 100 nucleotides in length, or in some aspects of the invention up to 1-2 kilobases or more in length, allows the formation of a duplex molecule that is both stable and selective. Molecules having complementary sequences over contiguous stretches greater than 20 bases in length are generally preferred, to increase stability and/or selectivity of the hybrid molecules obtained. One will generally prefer to design nucleic acid molecules for hybridization having one or more complementary sequences of 20 to 30 nucleotides, or even longer where desired. Such fragments may be readily prepared, for example, by directly synthesizing the fragment by chemical means or by introducing selected sequences into recombinant vectors for recombinant production.



[0123] Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNAs and/or RNAs or to provide primers for amplification of DNA or RNA from samples. Depending on the application envisioned, one would desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of the probe or primers for the target sequence.

[0124] For applications requiring high selectivity, one will typically desire to employ relatively high stringency conditions to form the hybrids. For example, relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.10 M NaCl at temperatures of about 50°C to about 70°C. Such high stringency conditions tolerate little, if any, mismatch between the probe or primers and the template or target strand and would be particularly suitable for isolating specific genes or for detecting specific mRNA transcripts. It is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide.

[0125] For certain applications, for example, site-directed mutagenesis, it is appreciated that lower stringency conditions are preferred. Under these conditions, hybridization may occur even though the sequences of the hybridizing strands are not perfectly complementary, but are mismatched at one or more positions. Conditions may be rendered less stringent by increasing salt concentration and/or decreasing temperature. For example, a medium stringency condition could be provided by about 0.1 to 0.25 M NaCl at temperatures of about 37°C to about 55°C, while a low stringency condition could be provided by about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Hybridization conditions can be readily manipulated depending on the desired results.

[0126] In other embodiments, hybridization may be achieved under conditions of, for example, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol, at temperatures between approximately 20°C to about 37°C. Other hybridization conditions utilized could include approximately 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, at temperatures ranging from approximately 40°C to about 72°C.

[0127] In certain embodiments, it will be advantageous to employ nucleic acids of defined sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of being detected. In preferred embodiments, one may

desire to employ a fluorescent label or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a detection means that is visibly or spectrophotometrically detectable, to identify specific hybridization with complementary nucleic acid containing samples.

[0128] In general, it is envisioned that the probes or primers described herein will be useful as reagents in solution hybridization, as in PCR™, for detection of expression of corresponding genes, as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to hybridization with selected probes under desired conditions. The conditions selected will depend on the particular circumstances (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, *etc.*). Optimization of hybridization conditions for the particular application of interest is well known to those of skill in the art. After washing of the hybridized molecules to remove non-specifically bound probe molecules, hybridization is detected, and/or quantified, by determining the amount of bound label. Representative solid phase hybridization methods are disclosed in U.S. Patent Nos. 5,843,663, 5,900,481 and 5,919,626. Other methods of hybridization that may be used in the practice of the present invention are disclosed in U.S. Patent Nos. 5,849,481, 5,849,486 and 5,851,772. The relevant portions of these and other references identified in this section of the Specification are incorporated herein by reference.

## **B. Amplification of Nucleic Acids**

[0129] Nucleic acids used as a template for amplification may be isolated from cells, tissues or other samples according to standard methodologies (Sambrook *et al.*, 1989). In certain embodiments, analysis is performed on whole cell or tissue homogenates or biological fluid samples without substantial purification of the template nucleic acid. The nucleic acid may be genomic DNA or fractionated or whole cell RNA. Where RNA is used, it may be desired to first convert the RNA to a complementary DNA.

[0130] The term "primer," as used herein, is meant to encompass any nucleic acid that is capable of priming the synthesis of a nascent nucleic acid in a template-dependent process. Typically, primers are oligonucleotides from ten to twenty and/or thirty base pairs in

length, but longer sequences can be employed. Primers may be provided in double-stranded and/or single-stranded form, although the single-stranded form is preferred.

[0131] Pairs of primers designed to selectively hybridize to nucleic acids corresponding to estrogen receptor alpha wildtype or mutant are contacted with the template nucleic acid under conditions that permit selective hybridization. Depending upon the desired application, high stringency hybridization conditions may be selected that will only allow hybridization to sequences that are completely complementary to the primers. In other embodiments, hybridization may occur under reduced stringency to allow for amplification of nucleic acids contain one or more mismatches with the primer sequences. Once hybridized, the template-primer complex is contacted with one or more enzymes that facilitate template-dependent nucleic acid synthesis. Multiple rounds of amplification, also referred to as "cycles," are conducted until a sufficient amount of amplification product is produced.

[0132] The amplification product may be detected or quantified. In certain applications, the detection may be performed by visual means. Alternatively, the detection may involve indirect identification of the product via chemiluminescence, radioactive scintigraphy of incorporated radiolabel or fluorescent label or even *via* a system using electrical and/or thermal impulse signals (Affymax technology; Bellus, 1994).

[0133] A number of template dependent processes are available to amplify the oligonucleotide sequences present in a given template sample. One of the best known amplification methods is the polymerase chain reaction (referred to as PCR<sup>TM</sup>) which is described in detail in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in Innis *et al.*, 1990, each of which is incorporated herein by reference in their entirety.

[0134] A reverse transcriptase PCR<sup>TM</sup> amplification procedure may be performed to quantify the amount of mRNA amplified. Methods of reverse transcribing RNA into cDNA are well known and described in Sambrook *et al.*, 1989. Alternative methods for reverse transcription utilize thermostable DNA polymerases. These methods are described in WO 90/07641. Polymerase chain reaction methodologies are well known in the art. Representative methods of RT-PCR are described in U.S. Patent No. 5,882,864.

[0135] Another method for amplification is ligase chain reaction ("LCR"), disclosed in European Application No. 320 308, incorporated herein by reference in its entirety. U.S. Patent 4,883,750 describes a method similar to LCR for binding probe pairs to a target sequence. A method based on PCR<sup>TM</sup> and oligonucleotide ligase assay (OLA), disclosed in U.S. Patent 5,912,148, may also be used.

[0136] Alternative methods for amplification of target nucleic acid sequences that may be used in the practice of the present invention are disclosed in U.S. Patent Nos. 5,843,650, 5,846,709, 5,846,783, 5,849,546, 5,849,497, 5,849,547, 5,858,652, 5,866,366, 5,916,776, 5,922,574, 5,928,905, 5,928,906, 5,932,451, 5,935,825, 5,939,291 and 5,942,391, GB Application No. 2 202 328, and in PCT Application No. PCT/US89/01025, each of which is incorporated herein by reference in its entirety.

[0137] Qbeta Replicase, described in PCT Application No. PCT/US87/00880, may also be used as an amplification method in the present invention. In this method, a replicative sequence of RNA that has a region complementary to that of a target is added to a sample in the presence of an RNA polymerase. The polymerase will copy the replicative sequence which may then be detected.

[0138] An isothermal amplification method, in which restriction endonucleases and ligases are used to achieve the amplification of target molecules that contain nucleotide 5'-[alpha-thio]-triphosphates in one strand of a restriction site may also be useful in the amplification of nucleic acids in the present invention (Walker *et al.*, 1992). Strand Displacement Amplification (SDA), disclosed in U.S. Patent No. 5,916,779, is another method of carrying out isothermal amplification of nucleic acids which involves multiple rounds of strand displacement and synthesis, *i.e.*, nick translation.

[0139] Other nucleic acid amplification procedures include transcription-based amplification systems (TAS), including nucleic acid sequence based amplification (NASBA) and 3SR (Kwoh *et al.*, 1989; Gingeras *et al.*, PCT Application WO 88/10315, incorporated herein by reference in their entirety). Davey *et al.*, European Application No. 329 822 disclose a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA), which may be used in accordance with the present invention.

[0140] Miller *et al.*, PCT Application WO 89/06700 (incorporated herein by reference in its entirety) disclose a nucleic acid sequence amplification scheme based on the hybridization of a promoter region/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. This scheme is not cyclic, *i.e.*, new templates are not produced from the resultant RNA transcripts. Other amplification methods include "race" and "one-sided PCR" (Frohman, 1990; Ohara *et al.*, 1989).

### C. Detection of Nucleic Acids

[0141] Following any amplification, it may be desirable to separate the amplification product from the template and/or the excess primer. In one embodiment, amplification products are separated by agarose, agarose-acrylamide or polyacrylamide gel electrophoresis using standard methods (Sambrook *et al.*, 1989). Separated amplification products may be cut out and eluted from the gel for further manipulation. Using low melting point agarose gels, the separated band may be removed by heating the gel, followed by extraction of the nucleic acid.

[0142] Separation of nucleic acids may also be effected by chromatographic techniques known in art. There are many kinds of chromatography which may be used in the practice of the present invention, including adsorption, partition, ion-exchange, hydroxylapatite, molecular sieve, reverse-phase, column, paper, thin-layer, and gas chromatography as well as HPLC.

[0143] In certain embodiments, the amplification products are visualized. A typical visualization method involves staining of a gel with ethidium bromide and visualization of bands under UV light. Alternatively, if the amplification products are integrally labeled with radio- or fluorometrically-labeled nucleotides, the separated amplification products can be exposed to x-ray film or visualized under the appropriate excitatory spectra.

[0144] In one embodiment, following separation of amplification products, a labeled nucleic acid probe is brought into contact with the amplified marker sequence. The probe preferably is conjugated to a chromophore but may be radiolabeled. In another embodiment, the probe is conjugated to a binding partner, such as an antibody or biotin, or another binding partner carrying a detectable moiety.

[0145] In particular embodiments, detection is by Southern blotting and hybridization with a labeled probe. The techniques involved in Southern blotting are well known to those of skill in the art. See Sambrook *et al.*, 1989. One example of the foregoing is described in U.S. Patent No. 5,279,721, incorporated by reference herein, which discloses an apparatus and method for the automated electrophoresis and transfer of nucleic acids. The apparatus permits electrophoresis and blotting without external manipulation of the gel and is ideally suited to carrying out methods according to the present invention.

[0146] Other methods of nucleic acid detection that may be used in the practice of the instant invention are disclosed in U.S. Patent Nos. 5,840,873, 5,843,640, 5,843,651,

5,846,708, 5,846,717, 5,846,726, 5,846,729, 5,849,487, 5,853,990, 5,853,992, 5,853,993, 5,856,092, 5,861,244, 5,863,732, 5,863,753, 5,866,331, 5,905,024, 5,910,407, 5,912,124, 5,912,145, 5,919,630, 5,925,517, 5,928,862, 5,928,869, 5,929,227, 5,932,413 and 5,935,791, each of which is incorporated herein by reference.

#### **D. Other Assays**

[0147] Other methods for genetic screening may be used within the scope of the present invention, for example, to detect mutations in genomic DNA, cDNA and/or RNA samples. Methods used to detect point mutations include denaturing gradient gel electrophoresis ("DGGE"), restriction fragment length polymorphism analysis ("RFLP"), chemical or enzymatic cleavage methods, direct sequencing of target regions amplified by PCR<sup>TM</sup> (see above), single-strand conformation polymorphism analysis ("SSCP") and other methods well known in the art.

[0148] One method of screening for point mutations is based on RNase cleavage of base pair mismatches in RNA/DNA or RNA/RNA heteroduplexes. As used herein, the term "mismatch" is defined as a region of one or more unpaired or mispaired nucleotides in a double-stranded RNA/RNA, RNA/DNA or DNA/DNA molecule. This definition thus includes mismatches due to insertion/deletion mutations, as well as single or multiple base point mutations.

[0149] U.S. Patent No. 4,946,773 describes an RNase A mismatch cleavage assay that involves annealing single-stranded DNA or RNA test samples to an RNA probe, and subsequent treatment of the nucleic acid duplexes with RNase A. For the detection of mismatches, the single-stranded products of the RNase A treatment, electrophoretically separated according to size, are compared to similarly treated control duplexes. Samples containing smaller fragments (cleavage products) not seen in the control duplex are scored as positive.

[0150] Other investigators have described the use of RNase I in mismatch assays. The use of RNase I for mismatch detection is described in literature from Promega Biotech. Promega markets a kit containing RNase I that is reported to cleave three out of four known mismatches. Others have described using the MutS protein or other DNA-repair enzymes for detection of single-base mismatches.

[0151] Alternative methods for detection of deletion, insertion or substitution mutations that may be used in the practice of the present invention are disclosed in U.S.

Patent Nos. 5,849,483, 5,851,770, 5,866,337, 5,925,525 and 5,928,870, each of which is incorporated herein by reference in its entirety.

#### **E. Kits**

**[0152]** All the essential materials and/or reagents required for detecting estrogen receptor alpha wildtype or mutant sequences in a sample may be assembled together in a kit. This generally will comprise a probe or primers designed to hybridize specifically to individual nucleic acids of interest in the practice of the present invention, including estrogen receptor alpha wildtype or mutant sequences. Also included may be enzymes suitable for amplifying nucleic acids, including various polymerases (reverse transcriptase, *Taq*, etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification. Such kits may also include enzymes and other reagents suitable for detection of specific nucleic acids or amplification products. Such kits generally will comprise, in suitable means, distinct containers for each individual reagent or enzyme as well as for each probe or primer pair.

#### **X. Estrogen Receptor $\alpha$ Nucleic Acids**

**[0153]** In a preferred embodiment, an estrogen receptor alpha nucleic acid sequence of the present invention contains an A908G mutation.

**[0154]** In specific embodiments, examples of the estrogen receptor alpha nucleic acid sequences which may include the A908G mutation include NM\_000125.1 (SEQ ID NO:1); AF242866 (SEQ ID NO:2); AF123496.1 (SEQ ID NO:3); AF120105 (SEQ ID NO:4); U47678.1 (SEQ ID NO:5); M12674.1 (SEQ ID NO:6); X03635.1 (SEQ ID NO:7); AF309825 (SEQ ID NO:19); AF061181 (SEQ ID NO:20); AF184588 (SEQ ID NO:21); AF181077 (SEQ ID NO:23); Z37167 (SEQ ID NO:24); AF173235 (SEQ ID NO:25); X90668 (SEQ ID NO:27); and AK025747 (SEQ ID NO:28). In other specific embodiments, examples of the estrogen receptor alpha amino acid sequences which may include the K303R substitution include NP\_000116.1 (SEQ ID NO:9); AAF65451.1 (SEQ ID NO:10); AAD23565.1 (SEQ ID NO:11); AAB00115.1 (SEQ ID NO:12); AAA52399.1 (SEQ ID NO:13); CAA27284.1 (SEQ ID NO:14); AAF00503.1 (SEQ ID NO:29); AAD53956.1 (SEQ ID NO:30); CAA85524.1 (SEQ ID NO:31); and BAB15231.1 (SEQ ID NO:32).

[0155] The term “estrogen receptor alpha wildtype or mutant sequence” as used herein refers respectively to the estrogen receptor alpha wildtype sequence or to a mutant sequence, wherein the mutant sequence comprises an A908G mutation.

#### A. Nucleic Acids and Uses Thereof

[0156] Certain aspects of the present invention concern at least one estrogen receptor alpha wildtype and/or mutant nucleic acid. In certain aspects, the at least one estrogen receptor alpha wildtype and/or mutant nucleic acid comprises a wild-type or mutant estrogen receptor alpha wildtype and/or mutant nucleic acid. In certain aspects, the estrogen receptor alpha wildtype and/or mutant nucleic acid comprises at least one transcribed nucleic acid. In particular aspects, the estrogen receptor alpha wildtype and/or mutant nucleic acid encodes at least one estrogen receptor alpha wildtype and/or mutant protein, polypeptide or peptide, or biologically functional equivalent thereof. In other aspects, the estrogen receptor alpha wildtype and/or mutant nucleic acid comprises at least one nucleic acid segment of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:28, or at least one biologically functional equivalent thereof.

[0157] The present invention also concerns the isolation or creation of at least one recombinant construct or at least one recombinant host cell through the application of recombinant nucleic acid technology known to those of skill in the art or as described herein. The recombinant construct or host cell may comprise at least one estrogen receptor alpha wildtype or mutant nucleic acid, and may express at least one estrogen receptor alpha wildtype or mutant protein, peptide or peptide, or at least one biologically functional equivalent thereof.

[0158] As used herein "wild-type" refers to the naturally occurring sequence of a nucleic acid at a genetic locus in the genome of an organism, and sequences transcribed or translated from such a nucleic acid. Thus, the term "wild-type" also may refer to the amino acid sequence encoded by the nucleic acid. As a genetic locus may have more than one sequence or alleles in a population of individuals, the term "wild-type" encompasses all such naturally occurring alleles. As used herein the term "polymorphic" means that variation exists (*i.e.* two or more alleles exist) at a genetic locus in the individuals of a population. As



used herein "mutant" refers to a change in the sequence of a nucleic acid or its encoded protein, polypeptide or peptide that is the result of the hand of man.

[0159] A nucleic acid may be made by any technique known to one of ordinary skill in the art. Non-limiting examples of synthetic nucleic acid, particularly a synthetic oligonucleotide, include a nucleic acid made by in vitro chemically synthesis using phosphotriester, phosphite or phosphoramidite chemistry and solid phase techniques such as described in EP 266,032, incorporated herein by reference, or via deoxynucleoside H-phosphonate intermediates as described by Froehler *et al.*, 1986, and U.S. Patent Serial No. 5,705,629, each incorporated herein by reference. A non-limiting example of enzymatically produced nucleic acid include one produced by enzymes in amplification reactions such as PCR<sup>TM</sup> (see for example, U.S. Patent 4,683,202 and U.S. Patent 4,682,195, each incorporated herein by reference), or the synthesis of oligonucleotides described in U.S. Patent No. 5,645,897, incorporated herein by reference. A non-limiting example of a biologically produced nucleic acid includes recombinant nucleic acid production in living cells, such as recombinant DNA vector production in bacteria (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

[0160] A nucleic acid may be purified on polyacrylamide gels, cesium chloride centrifugation gradients, or by any other means known to one of ordinary skill in the art (see for example, Sambrook *et al.* 1989, incorporated herein by reference).

[0161] The term "nucleic acid" will generally refer to at least one molecule or strand of DNA, RNA or a derivative or mimic thereof, comprising at least one nucleobase, such as, for example, a naturally occurring purine or pyrimidine base found in DNA (*e.g.* adenine "A," guanine "G," thymine "T" and cytosine "C") or RNA (*e.g.* A, G, uracil "U" and C). The term "nucleic acid" encompass the terms "oligonucleotide" and "polynucleotide." The term "oligonucleotide" refers to at least one molecule of between about 3 and about 100 nucleobases in length. The term "polynucleotide" refers to at least one molecule of greater than about 100 nucleobases in length. These definitions generally refer to at least one single-stranded molecule, but in specific embodiments will also encompass at least one additional strand that is partially, substantially or fully complementary to the at least one single-stranded molecule. Thus, a nucleic acid may encompass at least one double-stranded molecule or at least one triple-stranded molecule that comprises one or more complementary strand(s) or "complement(s)" of a particular sequence comprising a strand of the molecule. As used herein, a single stranded nucleic acid may be denoted by the prefix "ss", a double stranded nucleic acid by the prefix "ds", and a triple stranded nucleic acid by the prefix "ts."

[0162] Thus, the present invention also encompasses at least one nucleic acid that is complementary to a estrogen receptor alpha wildtype or mutant nucleic acid. In particular embodiments the invention encompasses at least one nucleic acid or nucleic acid segment complementary to the sequence set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28. Nucleic acid(s) that are "complementary" or "complement(s)" are those that are capable of base-pairing according to the standard Watson-Crick, Hoogsteen or reverse Hoogsteen binding complementarity rules. As used herein, the term "complementary" or "complement(s)" also refers to nucleic acid(s) that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above. The term "substantially complementary" refers to a nucleic acid comprising at least one sequence of consecutive nucleobases, or semiconsecutive nucleobases if one or more nucleobase moieties are not present in the molecule, are capable of hybridizing to at least one nucleic acid strand or duplex even if less than all nucleobases do not base pair with a counterpart nucleobase. In certain embodiments, a "substantially complementary" nucleic acid contains at least one sequence in which about 70%, about 71%, about 72%, about 73%, about 74%, about 75%, about 76%, about 77%, about 77%, about 78%, about 79%, about 80%, about 81%, about 82%, about 83%, about 84%, about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, to about 100%, and any range therein, of the nucleobase sequence is capable of base-pairing with at least one single or double stranded nucleic acid molecule during hybridization. In certain embodiments, the term "substantially complementary" refers to at least one nucleic acid that may hybridize to at least one nucleic acid strand or duplex in stringent conditions. In certain embodiments, a "partly complementary" nucleic acid comprises at least one sequence that may hybridize in low stringency conditions to at least one single or double stranded nucleic acid, or contains at least one sequence in which less than about 70% of the nucleobase sequence is capable of base-pairing with at least one single or double stranded nucleic acid molecule during hybridization.

[0163] As used herein, "hybridization", "hybridizes" or "capable of hybridizing" is understood to mean the forming of a double or triple stranded molecule or a molecule with partial double or triple stranded nature. The term "hybridization", "hybridize(s)" or "capable

of hybridizing" encompasses the terms "stringent condition(s)" or "high stringency" and the terms "low stringency" or "low stringency condition(s)."

[0164] As used herein "stringent condition(s)" or "high stringency" are those that allow hybridization between or within one or more nucleic acid strand(s) containing complementary sequence(s), but precludes hybridization of random sequences. Stringent conditions tolerate little, if any, mismatch between a nucleic acid and a target strand. Such conditions are well known to those of ordinary skill in the art, and are preferred for applications requiring high selectivity. Non-limiting applications include isolating at least one nucleic acid, such as a gene or nucleic acid segment thereof, or detecting at least one specific mRNA transcript or nucleic acid segment thereof, and the like.

[0165] Stringent conditions may comprise low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50°C to about 70°C. It is understood that the temperature and ionic strength of a desired stringency are determined in part by the length of the particular nucleic acid(s), the length and nucleobase content of the target sequence(s), the charge composition of the nucleic acid(s), and to the presence of formamide, tetramethylammonium chloride or other solvent(s) in the hybridization mixture. It is generally appreciated that conditions may be rendered more stringent, such as, for example, the addition of increasing amounts of formamide.

[0166] It is also understood that these ranges, compositions and conditions for hybridization are mentioned by way of non-limiting example only, and that the desired stringency for a particular hybridization reaction is often determined empirically by comparison to one or more positive or negative controls. Depending on the application envisioned it is preferred to employ varying conditions of hybridization to achieve varying degrees of selectivity of the nucleic acid(s) towards target sequence(s). In a non-limiting example, identification or isolation of related target nucleic acid(s) that do not hybridize to a nucleic acid under stringent conditions may be achieved by hybridization at low temperature and/or high ionic strength. Such conditions are termed "low stringency" or "low stringency conditions", and non-limiting examples of low stringency include hybridization performed at about 0.15 M to about 0.9 M NaCl at a temperature range of about 20°C to about 50°C. Of course, it is within the skill of one in the art to further modify the low or high stringency conditions to suite a particular application.

[0167] One or more nucleic acid(s) may comprise, or be composed entirely of, at least one derivative or mimic of at least one nucleobase, a nucleobase linker moiety and/or backbone moiety that may be present in a naturally occurring nucleic acid. As used herein a

"derivative" refers to a chemically modified or altered form of a naturally occurring molecule, while the terms "mimic" or "analog" refers to a molecule that may or may not structurally resemble a naturally occurring molecule, but functions similarly to the naturally occurring molecule. As used herein, a "moiety" generally refers to a smaller chemical or molecular component of a larger chemical or molecular structure, and is encompassed by the term "molecule."

**[0168]** As used herein a "nucleobase" refers to a naturally occurring heterocyclic base, such as A, T, G, C or U ("naturally occurring nucleobase(s)"), found in at least one naturally occurring nucleic acid (*i.e.* DNA and RNA), and their naturally or non-naturally occurring derivatives and mimics. Non-limiting examples of nucleobases include purines and pyrimidines, as well as derivatives and mimics thereof, which generally can form one or more hydrogen bonds ("anneal" or "hybridize") with at least one naturally occurring nucleobase in manner that may substitute for naturally occurring nucleobase pairing (*e.g.* the hydrogen bonding between A and T, G and C, and A and U).

**[0169]** Nucleobase, nucleoside and nucleotide mimics or derivatives are well known in the art, and have been described in exemplary references such as, for example, Scheit, Nucleotide Analogs (John Wiley, New York, 1980), incorporated herein by reference. "Purine" and "pyrimidine" nucleobases encompass naturally occurring purine and pyrimidine nucleobases and also derivatives and mimics thereof, including but not limited to, those purines and pyrimidines substituted by one or more of alkyl, caboxyalkyl, amino, hydroxyl, halogen (*i.e.* fluoro, chloro, bromo, or iodo), thiol, or alkylthiol wherein the alkyl group comprises of from about 1, about 2, about 3, about 4, about 5, to about 6 carbon atoms. Non-limiting examples of purines and pyrimidines include deazapurines, 2,6-diaminopurine, 5-fluorouracil, xanthine, hypoxanthine, 8-bromoguanine, 8-chloroguanine, bromothymine, 8-aminoguanine, 8-hydroxyguanine, 8-methylguanine, 8-thioguanine, azaguanines, 2-aminopurine, 5-ethylcytosine, 5-methylcyosine, 5-bromouracil, 5-ethyluracil, 5-iodouracil, 5-chlorouracil, 5-propyluracil, thiouracil, 2-methyladenine, methylthioadenine, N,N-diemethyladenine, azaadenines, 8-bromoadenine, 8-hydroxyadenine, 6-hydroxyaminopurine, 6-thiopurine, 4-(6-aminoheptyl/cytosine), and the like.

**[0170]** As used herein, "nucleoside" refers to an individual chemical unit comprising a nucleobase covalently attached to a nucleobase linker moiety. A non-limiting example of a "nucleobase linker moiety" is a sugar comprising 5-carbon atoms (a "5-carbon sugar"), including but not limited to deoxyribose, ribose or arabinose, and derivatives or mimics of 5-carbon sugars. Non-limiting examples of derivatives or mimics of 5-carbon

sugars include 2'-fluoro-2'-deoxyribose or carbocyclic sugars where a carbon is substituted for the oxygen atom in the sugar ring. By way of non-limiting example, nucleosides comprising purine (*i.e.* A and G) or 7-deazapurine nucleobases typically covalently attach the 9 position of the purine or 7-deazapurine to the 1'-position of a 5-carbon sugar. In another non-limiting example, nucleosides comprising pyrimidine nucleobases (*i.e.* C, T or U) typically covalently attach the 1 position of the pyrimidine to 1'-position of a 5-carbon sugar (Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992). However, other types of covalent attachments of a nucleobase to a nucleobase linker moiety are known in the art, and non-limiting examples are described herein.

[0171] As used herein, a "nucleotide" refers to a nucleoside further comprising a "backbone moiety" generally used for the covalent attachment of one or more nucleotides to another molecule or to each other to form one or more nucleic acids. The "backbone moiety" in naturally occurring nucleotides typically comprises a phosphorus moiety, which is covalently attached to a 5-carbon sugar. The attachment of the backbone moiety typically occurs at either the 3'- or 5'-position of the 5-carbon sugar. However, other types of attachments are known in the art, particularly when the nucleotide comprises derivatives or mimics of a naturally occurring 5-carbon sugar or phosphorus moiety, and non-limiting examples are described herein.

[0172] A non-limiting example of a nucleic acid comprising such nucleoside or nucleotide derivatives and mimics is a "polyether nucleic acid", described in U.S. Patent Serial No. 5,908,845, incorporated herein by reference, wherein one or more nucleobases are linked to chiral carbon atoms in a polyether backbone. Another example of a nucleic acid comprising nucleoside or nucleotide derivatives or mimics is a "peptide nucleic acid", also known as a "PNA", "peptide-based nucleic acid mimics" or "PENAMs", described in U.S. Patent Serial Nos. 5,786,461, 5,891,625, 5,773,571, 5,766,855, 5,736,336, 5,719,262, 5,714,331, 5,539,082, and WO 92/20702, each of which is incorporated herein by reference. A peptide nucleic acid generally comprises at least one nucleobase and at least one nucleobase linker moiety that is either not a 5-carbon sugar and/or at least one backbone moiety that is not a phosphate backbone moiety. Examples of nucleobase linker moieties described for PNAs include aza nitrogen atoms, amido and/or ureido tethers (see for example, U.S. Patent No. 5,539,082). Examples of backbone moieties described for PNAs include an aminoethylglycine, polyamide, polyethyl, polythioamide, polysulfonamide or polysulfonamide backbone moiety.

[0173] Peptide nucleic acids generally have enhanced sequence specificity, binding properties, and resistance to enzymatic degradation in comparison to molecules such as DNA and RNA (Egholm *et al.*, Nature 1993, 365, 566; PCT/EP/01219). In addition, U.S. Patent Nos. 5,766,855, 5,719,262, 5,714,331 and 5,736,336 describe PNAs comprising naturally and non-naturally occurring nucleobases and alkylamine side chains with further improvements in sequence specificity, solubility and binding affinity. These properties promote double or triple helix formation between a target nucleic acid and the PNA.

[0174] U.S. Patent No. 5,641,625 describes that the binding of a PNA may to a target sequence has applications the creation of PNA probes to nucleotide sequences, modulating (*i.e.* enhancing or reducing) gene expression by binding of a PNA to an expressed nucleotide sequence, and cleavage of specific dsDNA molecules. In certain embodiments, nucleic acid analogues such as one or more peptide nucleic acids may be used to inhibit nucleic acid amplification, such as in PCR, to reduce false positives and discriminate between single base mutants, as described in U.S. Patent Serial No. 5891,625.

[0175] U.S. Patent 5,786,461 describes PNAs with amino acid side chains attached to the PNA backbone to enhance solubility. The neutrality of the PNA backbone may contribute to the thermal stability of PNA/DNA and PNA/RNA duplexes by reducing charge repulsion. The melting temperature of PNA containing duplexes, or temperature at which the strands of the duplex release into single stranded molecules, has been described as less dependent upon salt concentration.

[0176] One method for increasing amount of cellular uptake property of PNAs is to attach a lipophilic group. U.S. application Ser. No. 117,363, filed Sep. 3, 1993, describes several alkylamino functionalities and their use in the attachment of such pendant groups to oligonucleosides. U.S. application Ser. No. 07/943,516, filed Sep. 11, 1992, and its corresponding published PCT application WO 94/06815, describe other novel amine-containing compounds and their incorporation into oligonucleotides for, inter alia, the purposes of enhancing cellular uptake, increasing lipophilicity, causing greater cellular retention and increasing the distribution of the compound within the cell.

[0177] Additional non-limiting examples of nucleosides, nucleotides or nucleic acids comprising 5-carbon sugar and/or backbone moiety derivatives or mimics are well known in the art.

[0178] In certain aspect, the present invention concerns at least one nucleic acid that is an isolated nucleic acid. As used herein, the term "isolated nucleic acid" refers to at least one nucleic acid molecule that has been isolated free of, or is otherwise free of, the bulk

of the total genomic and transcribed nucleic acids of one or more cells, particularly mammalian cells, and more particularly human cells. In certain embodiments, "isolated nucleic acid" refers to a nucleic acid that has been isolated free of, or is otherwise free of, bulk of cellular components and macromolecules such as lipids, proteins, small biological molecules, and the like. As different species may have a RNA or a DNA containing genome, the term "isolated nucleic acid" encompasses both the terms "isolated DNA" and "isolated RNA". Thus, the isolated nucleic acid may comprise a RNA or DNA molecule isolated from, or otherwise free of, the bulk of total RNA, DNA or other nucleic acids of a particular species. As used herein, an isolated nucleic acid isolated from a particular species is referred to as a "species specific nucleic acid." When designating a nucleic acid isolated from a particular species, such as human, such a type of nucleic acid may be identified by the name of the species. For example, a nucleic acid isolated from one or more humans would be an "isolated human nucleic acid", a nucleic acid isolated from human would be an "isolated human nucleic acid", and so forth.

[0179] Of course, more than one copy of an isolated nucleic acid may be isolated from biological material, or produced *in vitro*, using standard techniques that are known to those of skill in the art. In particular embodiments, the isolated nucleic acid is capable of expressing a protein, polypeptide or peptide that has the K303R substitution. In other embodiments, the isolated nucleic acid comprises an isolated estrogen receptor alpha wildtype or mutant nucleic acid sequence.

[0180] Herein certain embodiments, a "gene" refers to a nucleic acid that is transcribed. As used herein, a "gene segment" is a nucleic acid segment of a gene. In certain aspects, the gene includes regulatory sequences involved in transcription, or message production or composition. In particular embodiments, the gene comprises transcribed sequences that encode for a protein, polypeptide or peptide. In other particular aspects, the gene comprises an estrogen receptor alpha wildtype or mutant nucleic acid, and/or encodes an estrogen receptor alpha wildtype or mutant polypeptide or peptide coding sequences. In keeping with the terminology described herein, an "isolated gene" may comprise transcribed nucleic acid(s), regulatory sequences, coding sequences, or the like, isolated substantially away from other such sequences, such as other naturally occurring genes, regulatory sequences, polypeptide or peptide encoding sequences, *etc.* In this respect, the term "gene" is used for simplicity to refer to a nucleic acid comprising a nucleotide sequence that is transcribed, and the complement thereof. In particular aspects, the transcribed nucleotide sequence comprises at least one functional protein, polypeptide and/or peptide encoding unit.

As will be understood by those in the art, this function term "gene" includes both genomic sequences, RNA or cDNA sequences or smaller engineered nucleic acid segments, including nucleic acid segments of a non-transcribed part of a gene, including but not limited to the non-transcribed promoter or enhancer regions of a gene. Smaller engineered gene nucleic acid segments may express, or may be adapted to express using nucleic acid manipulation technology, proteins, polypeptides, domains, peptides, fusion proteins, mutants and/or such like.

**[0181]** "Isolated substantially away from other coding sequences" means that the gene of interest, in this case the estrogen receptor alpha gene(s) containing the A908G mutation, forms the significant part of the coding region of the nucleic acid, or that the nucleic acid does not contain large portions of naturally-occurring coding nucleic acids, such as large chromosomal fragments, other functional genes, RNA or cDNA coding regions. Of course, this refers to the nucleic acid as originally isolated, and does not exclude genes or coding regions later added to the nucleic acid by the hand of man.

**[0182]** In certain embodiments, the nucleic acid is a nucleic acid segment. As used herein, the term "nucleic acid segment", are smaller fragments of a nucleic acid, such as for non-limiting example, those that encode only part of the estrogen receptor alpha wildtype or mutant peptide or polypeptide sequence. In a preferred embodiment, the mutant peptide or polypeptide sequence comprises the K303R substitution. Thus, a "nucleic acid segment" may comprise any part of the estrogen receptor alpha wildtype or mutant gene sequence(s), of from about 2 nucleotides to the full length of the estrogen receptor alpha wildtype or mutant peptide or polypeptide encoding region. In certain embodiments, the "nucleic acid segment" encompasses the full length estrogen receptor alpha wildtype or mutant gene(s) sequence. In particular embodiments, the nucleic acid comprises any part of the SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28 sequence(s), of from about 2 nucleotides to the full length of the sequence disclosed in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28.

**[0183]** A non-limiting example of the present invention would be the generation of nucleic acid segments of various lengths and sequence composition for probes and primers based on the sequences disclosed in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3,



SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28.

**[0184]** The nucleic acid(s) of the present invention, regardless of the length of the sequence itself, may be combined with other nucleic acid sequences, including but not limited to, promoters, enhancers, polyadenylation signals, restriction enzyme sites, multiple cloning sites, coding segments, and the like, to create one or more nucleic acid construct(s). The length overall length may vary considerably between nucleic acid constructs. Thus, a nucleic acid segment of almost any length may be employed, with the total length preferably being limited by the ease of preparation or use in the intended recombinant nucleic acid protocol.

**[0185]** In a non-limiting example, one or more nucleic acid constructs may be prepared that include a contiguous stretch of nucleotides identical to or complementary to SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28. A nucleic acid construct may be about 3, about 5, about 8, about 10 to about 14, or about 15, about 20, about 30, about 40, about 50, about 100, about 200, about 500, about 1,000, about 2,000, about 3,000, about 5,000, about 10,000, about 15,000, about 20,000, about 30,000, about 50,000, about 100,000, about 250,000, about 500,000, about 750,000, to about 1,000,000 nucleotides in length, as well as constructs of greater size, up to and including chromosomal sizes (including all intermediate lengths and intermediate ranges), given the advent of nucleic acids constructs such as a yeast artificial chromosome are known to those of ordinary skill in the art. It will be readily understood that "intermediate lengths" and "intermediate ranges", as used herein, means any length or range including or between the quoted values (*i.e.* all integers including and between such values). Non-limiting examples of intermediate lengths include about 11, about 12, about 13, about 16, about 17, about 18, about 19, *etc.*; about 21, about 22, about 23, *etc.*; about 31, about 32, *etc.*; about 51, about 52, about 53, *etc.*; about 101, about 102, about 103, *etc.*; about 151, about 152, about 153, *etc.*; about 1,001, about 1002, *etc.*; about 50,001, about 50,002, *etc.*; about 750,001, about 750,002, *etc.*; about 1,000,001, about 1,000,002, *etc.* Non-limiting examples of intermediate ranges include about 3 to about 32, about 150 to about 500,001, about 3,032 to about 7,145, about 5,000 to about 15,000, about 20,007 to about 1,000,003, *etc.*

[0186] In particular embodiments, the invention concerns one or more recombinant vector(s) comprising nucleic acid sequences that encode an estrogen receptor alpha wildtype or mutant protein, polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially as set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32 corresponding to human SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28. In other embodiments, the invention concerns recombinant vector(s) comprising nucleic acid sequences that encode a human estrogen receptor alpha wildtype or mutant protein, polypeptide or peptide that includes within its amino acid sequence a contiguous amino acid sequence in accordance with, or essentially as set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32. In particular aspects, the recombinant vectors are DNA vectors.

[0187] The term "a sequence essentially as set forth in SEQ ID NO:9" means that the sequence substantially corresponds to a portion of SEQ ID NO:9 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:9. Thus, "a sequence essentially as set forth in SEQ ID NO:1 encompasses nucleic acids, nucleic acid segments, and genes that comprise part or all of the nucleic acid sequences as set forth in SEQ ID NO:1.

[0188] The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein. Accordingly, a sequence that has between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32 will be a sequence that is "essentially as set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32", provided the biological activity of the protein, polypeptide or peptide is maintained.

[0189] In certain other embodiments, the invention concerns at least one recombinant vector that include within its sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID

NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28. In particular embodiments, the recombinant vector comprises DNA sequences that encode protein(s), polypeptide(s) or peptide(s) exhibiting estrogen receptor alpha wildtype or mutant activity.

**[0190]** The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine and serine, and also refers to codons that encode biologically equivalent amino acids, which are well known in the art.

**[0191]** Information on codon usage in a variety of non-human organisms is known in the art (see for example, Bennetzen and Hall, 1982; Ikemura, 1981a, 1981b, 1982; Grantham *et al.*, 1980, 1981; Wada *et al.*, 1990; each of these references are incorporated herein by reference in their entirety). Thus, it is contemplated that codon usage may be optimized for other animals, as well as other organisms such as fungi, plants, prokaryotes, virus and the like, as well as organelles that contain nucleic acids, such as mitochondria, chloroplasts and the like, based on the preferred codon usage as would be known to those of ordinary skill in the art.

**[0192]** It will also be understood that amino acid sequences or nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, or various combinations thereof, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein, polypeptide or peptide activity where expression of a proteinaceous composition is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' and/or 3' portions of the coding region or may include various internal sequences, *i.e.*, introns, which are known to occur within genes.

**[0193]** Excepting intronic and flanking regions, and allowing for the degeneracy of the genetic code, nucleic acid sequences that have between about 70% and about 79%; or more preferably, between about 80% and about 89%; or even more particularly, between about 90% and about 99%; of nucleotides that are identical to the nucleotides of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28 will be nucleic acid sequences that are "essentially as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ

ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28".

**[0194]** It will also be understood that this invention is not limited to the particular nucleic acid sequences of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:27, or SEQ ID NO:28, or the amino acid sequences of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32, respectively. Recombinant vectors and isolated nucleic acid segments may therefore variously include these coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, and they may encode larger polypeptides or peptides that nevertheless include such coding regions or may encode biologically functional equivalent proteins, polypeptide or peptides that have mutant amino acids sequences.

**[0195]** The nucleic acids of the present invention encompass biologically functional equivalent estrogen receptor alpha wildtype or mutant proteins, polypeptides, or peptides. Such sequences may arise as a consequence of codon redundancy or functional equivalency that are known to occur naturally within nucleic acid sequences or the proteins, polypeptides or peptides thus encoded. Alternatively, functionally equivalent proteins, polypeptides or peptides may be created via the application of recombinant DNA technology, in which changes in the protein, polypeptide or peptide structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced, for example, through the application of site-directed mutagenesis techniques as discussed herein below, *e.g.*, to introduce improvements or alterations to the antigenicity of the protein, polypeptide or peptide, or to test mutants in order to examine estrogen receptor alpha wildtype or mutant protein, polypeptide or peptide activity at the molecular level.

**[0196]** Fusion proteins, polypeptides or peptides may be prepared, *e.g.*, where the estrogen receptor alpha wildtype or mutant coding regions are aligned within the same expression unit with other proteins, polypeptides or peptides having desired functions. Non-limiting examples of such desired functions of expression sequences include purification or immunodetection purposes for the added expression sequences, *e.g.*, proteinaceous compositions that may be purified by affinity chromatography or the enzyme labeling of coding regions, respectively.

[0197] Encompassed by the invention are nucleic acid sequences encoding relatively small peptides or fusion peptides, such as, for example, peptides of from about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29, about 30, about 31, about 32, about 33, about 34, about 35, about 35, about 36, about 37, about 38, about 39, about 40, about 41, about 42, about 43, about 44, about 45, about 46, about 47, about 48, about 49, about 50, about 51, about 52, about 53, about 54, about 55, about 56, about 57, about 58, about 59, about 60, about 61, about 62, about 63, about 64, about 65, about 66, about 67, about 68, about 69, about 70, about 71, about 72, about 73, about 74, about 75, about 76, about 77, about 78, about 79, about 80, about 81, about 82, about 83, about 84, about 85, about 86, about 87, about 88, about 89, about 90, about 91, about 92, about 93, about 94, about 95, about 96, about 97, about 98, about 99, to about 100 amino acids in length, or more preferably, of from about 15 to about 30 amino acids in length; as set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32, and also larger polypeptides up to and including proteins corresponding to the full-length sequences set forth in SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, or SEQ ID NO:32.

[0198] As used herein an "organism" may be a prokaryote, eukaryote, virus and the like. As used herein the term "sequence" encompasses both the terms "nucleic acid" and "proteinaceous" or "proteinaceous composition." As used herein, the term "proteinaceous composition" encompasses the terms "protein", "polypeptide" and "peptide." As used herein "artificial sequence" refers to a sequence of a nucleic acid not derived from sequence naturally occurring at a genetic locus, as well as the sequence of any proteins, polypeptides or peptides encoded by such a nucleic acid. A "synthetic sequence", refers to a nucleic acid or proteinaceous composition produced by chemical synthesis *in vitro*, rather than enzymatic production *in vitro* (*i.e.* an "enzymatically produced" sequence) or biological production *in vivo* (*i.e.* a "biologically produced" sequence).

**XI. Protein Computer Modeling**

[0199] To determine whether a mutation would likely produce a protein, polypeptide or peptide with a less exposed site and/or motif, the putative location of the altered, moved or added site and/or sequence could be determined by comparison of the mutated sequence to that of the unmutated protein, polypeptide or peptide's secondary and tertiary structure, as determined by such methods known to those of ordinary skill in the art including, but not limited to, X-ray crystallography, NMR or computer modeling. Computer models of various polypeptide and peptide structures are also available in the literature or computer databases. In a non-limiting example, the Entrez database (<http://www.ncbi.nlm.nih.gov/Entrez/>) may be used by one of ordinary skill in the art to identify target sequences and regions for mutagenesis. The Entrez database is crosslinked to a database of 3-D structures for the identified amino acid sequence, if known. Such molecular models may be used to identify sites and/or flanking sequences in peptides and polypeptides that are more exposed to contact with external molecules, (e.g. receptors) than similar sequences embedded in the interior of the polypeptide or polypeptide. In certain embodiments, when adding at least one site and/or flanking sequence is desirable, regions of the protein that are more exposed to contact with external molecules are preferred as sites to add such a sequence. The mutated or wild-type protein, polypeptide or peptide's structure could be determined by X-ray crystallography or NMR directly before use in *in vitro* or *in vivo* assays, as would be known to one of ordinary skill in the art.

**XII. Prokaryotic Peptide Display**

[0200] Molecular analysis of naturally occurring and artificial protein libraries has been greatly improved by the development of various "display" methodologies. The general scheme behind display techniques is the advantageous expression of peptides, and their disposition on some biological surface (phage, cell, *etc.*). The ability of different version of the displaying organism to present millions and millions of different variants allows the rapid screening of the corresponding library for biological function.

[0201] In U.S. Patent 5,821,047, monovalent phage display is described. This method provides for the selection of novel proteins, and variants thereof. The method comprises fusing a gene encoding a protein of interest to the carboxy terminal domain of the gene III coat protein of the filamentous phage M13. The fusion is mutated to form a library

of structurally related fusion proteins that are expressed in low quantity on the surface of phagemid candidates.

[0202] U.S. Patent 5,571,698 describes directed evolution using an M13 phagemid system. A protein is expression as a fusion with the M13 gene III protein. Successive rounds of mutagenesis are performed, each time selecting for improved biological function, e.g., binding of a protein to a cognate binding partner.

[0203] Heterodimer phage libraries are described in U.S. Patent 5,759,817. Filamentous phage comprising a matrix of cpVIII proteins encapsulating a genome encoding first and second polypeptides of an autogenously assembling receptor, such as an antibody, are provided. The receptor is surface-integrated into the phage coat matrix via the cpVIII membrane anchor, presenting the receptor for biological assessment.

[0204] Another system, lambdoid phage, also can be used for display purposes. In U.S. Patent 5,672,024, lambdoid phage comprising a matrix of proteins encapsulating a genome encoding first and second polypeptides of an autogenously assembling receptor are prepared. The surface-integrated receptor is available on the surface on the phage for characterization.

[0205] Immunoglobulin heavy chain libraries are displayed by phage as described in U.S. Patent 5,824,520. A single chain antibody library is generated by creating highly divergent, synthetic hypervariable regions, followed by phage display and selection. The resulting antibodies were used to inhibit intracellular enzyme activity. Another patent describing antibody display is U.S. Patent 5,922,545.

[0206] Another example of phage display can be found in U.S. Patent 5,780,279. This method provides for the identification and selection of novel substrates for enzymes. The method comprises constructing a gene fusion comprising DNA encoding a polypeptide fused to a DNA encoding a substrate peptide, which in turn is fusion to DNA encoding at least a portion of a phage coat protein. The DNA encoding the substrate peptide is mutated at one or more codons, thereby generating a family of mutants. The fusion protein is expressed on the surface of the phagemid particle and subjected to chemical or enzymatic modification of the substrate peptide. Those phagemid particles that have been modified are then separated from those that have not.

[0207] Bacteria also have been used successfully to display proteins. U.S. Patent 5,348,867, describes expression of proteins on bacterial surfaces. The compositions and methods provide stable, surface-expressed polypeptide from recombinant gram-negative bacterial cell hosts. A tripartite chimeric gene and its related recombinant vector include

separate DNA sequences for directing or targeting and translocating a desired gene product from a cell periplasm to the external cell surface. A wide range of polypeptides may be efficiently surface expressed using this system. See also, U.S. Patents 5,508,192 and 5,866,344.

[0208] U.S. Patent 5,500,353 describes another bacterial display system. Bacteria (*e.g.*, *Caulobacter*) having a S-layer modified such that the bacterium S-layer protein gene contains one or more in-frame fusions coding for one or more heterologous peptides or polypeptides is described. The proteins are expressed on the surface of the bacterium, which may advantageously be cultured as a film.

### XIII. Rational Drug Design

[0209] The goal of rational drug design is to produce structural analogs of biologically active compounds. By creating such analogs, it is possible to fashion drugs which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for the antagonist of estrogen receptor alpha K303R polypeptide of the invention or a fragment thereof. This could be accomplished by X-ray crystallography, computer modeling or by a combination of both approaches. An alternative approach involves the random replacement of functional groups throughout the estrogen receptor alpha K303R polypeptide, and the resulting affect on function determined.

[0210] It also is possible to isolate a estrogen receptor alpha K303R polypeptide specific antibody, selected by a functional assay, and then solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

[0211] Thus, one may design drugs which have enhanced and improved biological activity, for example, anti-breast cancer activity relative to a starting compound.



By virtue of the chemical isolation procedures and descriptions well known in the art, sufficient amounts of the estrogen receptor alpha K303R polypeptide of the invention can be produced to perform crystallographic studies. In addition, knowledge of the chemical characteristics of these compounds permits computer employed predictions of structure-function relationships that facilitate drug design.

#### **XIV. Screening For Modulators Of the Protein Function**

[0212] The present invention further comprises methods for identifying modulators of the function of an estrogen receptor alpha K303R polypeptide. These assays may comprise random screening of large libraries of candidate substances; alternatively, the assays may be used to focus on particular classes of compounds selected with an eye towards structural attributes that are believed to make them more likely to modulate the function of estrogen receptor alpha K303R polypeptide.

[0213] By fuction, it is meant that one may assay for antagonist and/or agonist activity of an estrogen receptor alpha K303R polypeptide.

[0214] To identify a estrogen receptor alpha K303R polypeptide modulator, one generally will determine the function of estrogen receptor alpha K303R polypeptide in the presence and absence of the candidate substance, a modulator defined as any substance that alters function. For example, a method generally comprises:

- (a) providing a candidate modulator;
  - (b) admixing the candidate modulator with an isolated compound or cell, or a suitable experimental animal;
  - (c) measuring one or more characteristics of the compound, cell or animal in step (b); and
  - (d) comparing the characteristic measured in step (c) with the characteristic of the compound, cell or animal in the absence of said candidate modulator,
- wherein a difference between the measured characteristics indicates that said candidate modulator is, indeed, a modulator of the compound, cell or animal.

[0215] Assays may be conducted in cell free systems, in isolated cells, or in organisms including transgenic animals.

[0216] It will, of course, be understood that all the screening methods of the present invention are useful in themselves notwithstanding the fact that effective candidates

may not be found. The invention provides methods for screening for such candidates, not solely methods of finding them.

#### A. Modulators

[0217] As used herein the term "candidate substance" refers to any molecule that may potentially inhibit or enhance estrogen receptor alpha K303R polypeptide activity. The candidate substance may be a protein or fragment thereof, a small molecule, or even a nucleic acid molecule. It may prove to be the case that the most useful pharmacological compounds will be compounds that are structurally related to SERMs. Using lead compounds to help develop improved compounds is known as "rational drug design" and includes not only comparisons with known inhibitors and activators, but predictions relating to the structure of target molecules.

[0218] The goal of rational drug design is to produce structural analogs of biologically active polypeptides or target compounds. By creating such analogs, it is possible to fashion drugs, which are more active or stable than the natural molecules, which have different susceptibility to alteration or which may affect the function of various other molecules. In one approach, one would generate a three-dimensional structure for a target molecule, or a fragment thereof. This could be accomplished by x-ray crystallography, computer modeling or by a combination of both approaches.

[0219] It also is possible to use antibodies to ascertain the structure of a target compound activator or inhibitor. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of anti-idiotypic would be expected to be an analog of the original antigen. The anti-idiotypic could then be used to identify and isolate peptides from banks of chemically- or biologically-produced peptides. Selected peptides would then serve as the pharmacore. Anti-idiotypes may be generated using the methods described herein for producing antibodies, using an antibody as the antigen.

[0220] On the other hand, one may simply acquire, from various commercial sources, small molecule libraries that are believed to meet the basic criteria for useful drugs in an effort to "brute force" the identification of useful compounds. Screening of such libraries, including combinatorially generated libraries (*e.g.*, peptide libraries), is a rapid and efficient way to screen large number of related (and unrelated) compounds for activity.

Combinatorial approaches also lend themselves to rapid evolution of potential drugs by the creation of second, third and fourth generation compounds modeled of active, but otherwise undesirable compounds.

[0221] Candidate compounds may include fragments or parts of naturally-occurring compounds, or may be found as active combinations of known compounds, which are otherwise inactive. It is proposed that compounds isolated from natural sources, such as animals, bacteria, fungi, plant sources, including leaves and bark, and marine samples may be assayed as candidates for the presence of potentially useful pharmaceutical agents. It will be understood that the pharmaceutical agents to be screened could also be derived or synthesized from chemical compositions or man-made compounds. Thus, it is understood that the candidate substance identified by the present invention may be peptide, polypeptide, polynucleotide, small molecule inhibitors or any other compounds that may be designed through rational drug design starting from known inhibitors or stimulators.

[0222] Other suitable modulators include antisense molecules, ribozymes, and antibodies (including single chain antibodies), each of which would be specific for the target molecule. Such compounds are described in greater detail elsewhere in this document. For example, an antisense molecule that bound to a translational or transcriptional start site, or splice junctions, would be ideal candidate inhibitors.

[0223] In addition to the modulating compounds initially identified, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the structure of the modulators. Such compounds, which may include peptidomimetics of peptide modulators, may be used in the same manner as the initial modulators.

[0224] An inhibitor according to the present invention may be one which exerts its inhibitory or activating effect upstream, downstream or directly on an estrogen receptor alpha K303R polypeptide. Regardless of the type of inhibitor or activator identified by the present screening methods, the effect of the inhibition or activator by such a compound results in reduction in the activity of estrogen receptor alpha K303R polypeptide as a transcription factor as compared to that observed in the absence of the added candidate substance.

## **B. *In vitro* Assays**

[0225] A quick, inexpensive and easy assay to run is an *in vitro* assay. Such assays generally use isolated molecules, can be run quickly and in large numbers, thereby increasing the amount of information obtainable in a short period of time. A variety of vessels may be used to run the assays, including test tubes, plates, dishes and other surfaces such as dipsticks or beads.

[0226] One example of a cell free assay is a binding assay. While not directly addressing function, the ability of a modulator to bind to a target molecule in a specific fashion is strong evidence of a related biological effect. For example, binding of a molecule to a target may, in and of itself, be inhibitory, due to steric, allosteric or charge-charge interactions. The target may be either free in solution, fixed to a support, expressed in or on the surface of a cell. Either the target or the compound may be labeled, thereby permitting determining of binding. Usually, the target will be the labeled species, decreasing the chance that the labeling will interfere with or enhance binding. Competitive binding formats can be performed in which one of the agents is labeled, and one may measure the amount of free label versus bound label to determine the effect on binding.

[0227] A technique for high throughput screening of compounds is described in WO 84/03564. Large numbers of small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. Bound polypeptide is detected by various methods.

### C. *In cyto* Assays

[0228] The present invention also contemplates the screening of compounds for their ability to modulate estrogen receptor alpha K303R polypeptide in cells. Various cell lines can be utilized for such screening assays, including cells specifically engineered for this purpose. For example, cells comprising an estrogen receptor alpha K303R polypeptide-expressing vector, a vector comprising an estrogen regulatory element operatively linked to a reporter polynucleotide, and a compound to be screened are contemplated.

[0229] Depending on the assay, culture may be required. The cell is examined using any of a number of different physiologic assays. Alternatively, molecular analysis may be performed, for example, looking at protein expression, mRNA expression (including differential display of whole cell or polyA RNA) and others.

### D. *In vivo* Assays

[0230] *In vivo* assays involve the use of various animal models, including transgenic animals that have been engineered to have specific defects, or carry markers that can be used to measure the ability of a candidate substance to reach and effect different cells within the organism. Due to their size, ease of handling, and information on their physiology and genetic make-up, mice are a preferred embodiment, especially for transgenics. However, other animals are suitable as well, including rats, rabbits, hamsters, guinea pigs, gerbils, woodchucks, cats, dogs, sheep, goats, pigs, cows, horses and monkeys (including chimps, gibbons and baboons). Assays for modulators may be conducted using an animal model derived from any of these species.

[0231] In such assays, one or more candidate substances are administered to an animal, and the ability of the candidate substance(s) to alter one or more characteristics, as compared to a similar animal not treated with the candidate substance(s), identifies a modulator. The characteristics may be any of those discussed above with regard to the function of a particular compound (*e.g.*, enzyme, receptor, hormone) or cell (*e.g.*, growth, tumorigenicity, survival), or instead a broader indication such as behavior, anemia, immune response, etc.

[0232] The present invention provides methods of screening for a candidate substance that antagonizes an estrogen receptor alpha K303R polypeptide. In these embodiments, the present invention is directed to a method for determining the ability of a candidate substance to reduce the activity of estrogen receptor alpha K303R polypeptide, generally including the steps of: administering a candidate substance to the animal; and determining the ability of the candidate substance to reduce one or more characteristics of estrogen receptor alpha K303R polypeptide.

[0233] Treatment of these animals with test compounds will involve the administration of the compound, in an appropriate form, to the animal. Administration will be by any route that could be utilized for clinical or non-clinical purposes, including but not limited to oral, nasal, buccal, or even topical. Alternatively, administration may be by intratracheal instillation, bronchial instillation, intradermal, subcutaneous, intramuscular, intraperitoneal or intravenous injection. Specifically contemplated routes are systemic intravenous injection, regional administration *via* blood or lymph supply, or directly to an affected site.

[0234] Determining the effectiveness of a compound *in vivo* may involve a variety of different criteria. Also, measuring toxicity and dose response can be performed in animals in a more meaningful fashion than in *in vitro* or *in cyto* assays.

## XV. Mimetics

[0235] The present inventors contemplate that structurally similar compounds may be formulated to mimic the key portions of peptide or polypeptides of the present invention. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and, hence, also are functional equivalents.

[0236] Certain mimetics that mimic elements of protein secondary and tertiary structure are described in Johnson *et al.* (1993). The underlying rationale behind the use of peptide mimetics is that the peptide backbone of proteins exists chiefly to orient amino acid side chains in such a way as to facilitate molecular interactions, such as those of antibody and/or antigen. A peptide mimetic is thus designed to permit molecular interactions similar to the natural molecule.

[0237] Some successful applications of the peptide mimetic concept have focused on mimetics of  $\beta$ -turns within proteins, which are known to be highly antigenic. Likely  $\beta$ -turn structure within a polypeptide can be predicted by computer-based algorithms, as discussed herein. Once the component amino acids of the turn are determined, mimetics can be constructed to achieve a similar spatial orientation of the essential elements of the amino acid side chains.

[0238] Other approaches have focused on the use of small, multidisulfide-containing proteins as attractive structural templates for producing biologically active conformations that mimic the binding sites of large proteins. Vita *et al.* (1998). A structural motif that appears to be evolutionarily conserved in certain toxins is small (30-40 amino acids), stable, and high permissive for mutation. This motif is composed of a beta sheet and an alpha helix bridged in the interior core by three disulfides.

[0239] Beta II turns have been mimicked successfully using cyclic L-pentapeptides and those with D-amino acids. Weisshoff *et al.* (1999). Also, Johannesson *et al.* (1999) report on bicyclic tripeptides with reverse turn inducing properties.

[0240] Methods for generating specific structures have been disclosed in the art. For example, alpha-helix mimetics are disclosed in U.S. Patents 5,446,128; 5,710,245; 5,840,833; and 5,859,184. These structures render the peptide or protein more thermally stable, also increase resistance to proteolytic degradation. Six, seven, eleven, twelve, thirteen and fourteen membered ring structures are disclosed.

[0241] Methods for generating conformationally restricted beta turns and beta bulges are described, for example, in U.S. Patents 5,440,013; 5,618,914; and 5,670,155. Beta-turns permit changed side substituents without having changes in corresponding backbone conformation, and have appropriate termini for incorporation into peptides by standard synthesis procedures. Other types of mimetic turns include reverse and gamma turns. Reverse turn mimetics are disclosed in U.S. Patents 5,475,085 and 5,929,237, and gamma turn mimetics are described in U.S. Patents 5,672,681 and 5,674,976.

## XVI. Immunodetection Methods

[0242] In still further embodiments, the present invention concerns immunodetection methods for binding, purifying, removing, quantifying and/or otherwise generally detecting biological components such as estrogen receptor alpha protein or nucleic acid components. The estrogen receptor alpha antibodies prepared in accordance with the present invention may be employed to detect wild-type and/or mutant estrogen receptor alpha proteins, polypeptides and/or peptides. In specific embodiments, the antibodies detect an acetylated form of estrogen receptor alpha protein, polypeptide and/or peptide or the antibodies detect an A908G estrogen receptor alpha nucleic acid mutation. The use of wild-type and/or mutant estrogen receptor alpha specific antibodies is contemplated. Some immunodetection methods include enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunoradiometric assay, fluoroimmunoassay, chemiluminescent assay, bioluminescent assay, and Western blot to mention a few. The steps of various useful immunodetection methods have been described in the scientific literature, such as, *e.g.*, Doolittle MH and Ben-Zeev O, 1999; Gulbis B and Galand P, 1993; De Jager R *et al.*, 1993; and Nakamura *et al.*, 1987, each incorporated herein by reference.

[0243] In general, the immunobinding methods include obtaining a sample suspected of containing estrogen receptor alpha protein, polypeptide and/or peptide, and contacting the sample with a first anti-estrogen receptor alpha antibody in accordance with the present invention, as the case may be, under conditions effective to allow the formation of immunocomplexes.

[0244] These methods include methods for purifying wild-type and/or mutant estrogen receptor alpha proteins, polypeptides and/or peptides as may be employed in purifying wild-type and/or mutant estrogen receptor alpha proteins, polypeptides and/or peptides from patients' samples and/or for purifying recombinantly expressed wild-type or mutant estrogen receptor alpha proteins, polypeptides and/or peptides. In these instances, the

antibody removes the antigenic wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or peptide component from a sample. The antibody will preferably be linked to a solid support, such as in the form of a column matrix, and the sample suspected of containing the wild-type or mutant estrogen receptor alpha protein antigenic component will be applied to the immobilized antibody. The unwanted components will be washed from the column, leaving the antigen immunocomplexed to the immobilized antibody, which wild-type or mutant estrogen receptor alpha protein antigen is then collected by removing the wild-type or mutant estrogen receptor alpha protein and/or peptide from the column.

[0245] The immunobinding methods also include methods for detecting and quantifying the amount of a wild-type or mutant estrogen receptor alpha protein reactive component in a sample and the detection and quantification of any immune complexes formed during the binding process. Here, one would obtain a sample suspected of containing a wild-type or mutant estrogen receptor alpha protein and/or peptide, and contact the sample with an antibody against wild-type or mutant estrogen receptor alpha, and then detect and quantify the amount of immune complexes formed under the specific conditions.

[0246] In terms of antigen detection, the biological sample analyzed may be any sample that is suspected of containing a wild-type or mutant estrogen receptor alpha protein-specific antigen, such as a breast tissue section or specimen, a homogenized breast tissue extract, a breast cell, separated and/or purified forms of any of the above wild-type or mutant estrogen receptor alpha protein-containing compositions, or even any biological fluid that comes into contact with the breast tissue. Diseases that may be suspected of containing a wild-type or mutant estrogen receptor alpha protein-specific antigen include, but are not limited to, breast cancer.

[0247] Contacting the chosen biological sample with the antibody under effective conditions and for a period of time sufficient to allow the formation of immune complexes (primary immune complexes) is generally a matter of simply adding the antibody composition to the sample and incubating the mixture for a period of time long enough for the antibodies to form immune complexes with, *i.e.*, to bind to, any estrogen receptor alpha protein antigens present. After this time, the sample-antibody composition, such as a tissue section, ELISA plate, dot blot or western blot, will generally be washed to remove any non-specifically bound antibody species, allowing only those antibodies specifically bound within the primary immune complexes to be detected.

[0248] In general, the detection of immunocomplex formation is well known in the art and may be achieved through the application of numerous approaches. These methods



are generally based upon the detection of a label or marker, such as any of those radioactive, fluorescent, biological and enzymatic tags. U.S. Patents concerning the use of such labels include 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149 and 4,366,241, each incorporated herein by reference. Of course, one may find additional advantages through the use of a secondary binding ligand such as a second antibody and/or a biotin/avidin ligand binding arrangement, as is known in the art.

[0249] The estrogen receptor alpha antibody employed in the detection may itself be linked to a detectable label, wherein one would then simply detect this label, thereby allowing the amount of the primary immune complexes in the composition to be determined. Alternatively, the first antibody that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under effective conditions and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected.

[0250] Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a third binding ligand or antibody that has binding affinity for the second antibody, again under effective conditions and for a period of time sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if this is desired.

[0251] One method of immunodetection designed by Charles Cantor uses two different antibodies. A first step biotinylated, monoclonal or polyclonal antibody is used to detect the target antigen(s), and a second step antibody is then used to detect the biotin attached to the complexed biotin. In that method the sample to be tested is first incubated in a solution containing the first step antibody. If the target antigen is present, some of the antibody binds to the antigen to form a biotinylated antibody/antigen complex. The antibody/antigen complex is then amplified by incubation in successive solutions of

streptavidin (or avidin), biotinylated DNA, and/or complementary biotinylated DNA, with each step adding additional biotin sites to the antibody/antigen complex. The amplification steps are repeated until a suitable level of amplification is achieved, at which point the sample is incubated in a solution containing the second step antibody against biotin. This second step antibody is labeled, as for example with an enzyme that can be used to detect the presence of the antibody/antigen complex by histoenzymology using a chromogen substrate. With suitable amplification, a conjugate can be produced which is macroscopically visible.

[0252] Another known method of immunodetection takes advantage of the immuno-PCR (Polymerase Chain Reaction) methodology. The PCR method is similar to the Cantor method up to the incubation with biotinylated DNA, however, instead of using multiple rounds of streptavidin and biotinylated DNA incubation, the DNA/biotin/streptavidin/antibody complex is washed out with a low pH or high salt buffer that releases the antibody. The resulting wash solution is then used to carry out a PCR reaction with suitable primers with appropriate controls. At least in theory, the enormous amplification capability and specificity of PCR can be utilized to detect a single antigen molecule.

[0253] The immunodetection methods of the present invention have evident utility in the diagnosis and prognosis of conditions such as various forms of cancer, such as breast cancer. Here, a biological and/or clinical sample suspected of containing a wild-type or mutant estrogen receptor alpha protein, polypeptide, peptide and/or mutant is used. However, these embodiments also have applications to non-clinical samples, such as in the titering of antigen or antibody samples, for example in the selection of hybridomas.

[0254] In the clinical diagnosis and/or monitoring of patients with various forms of breast cancer, the detection of estrogen receptor alpha mutant, and/or an alteration in the levels of estrogen receptor alpha, in comparison to the levels in a corresponding biological sample from a normal subject is indicative of a patient with cancer, such as breast cancer. However, as is known to those of skill in the art, such a clinical diagnosis would not necessarily be made on the basis of this method in isolation. Those of skill in the art are very familiar with differentiating between significant differences in types and/or amounts of biomarkers, which represent a positive identification, and/or low level and/or background changes of biomarkers. Indeed, background expression levels are often used to form a "cut-off" above which increased detection will be scored as significant and/or positive.

#### **A. ELISAs**

[0255] As detailed above, immunoassays, in their most simple and/or direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and/or radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and/or western blotting, dot blotting, FACS analyses, and/or the like may also be used.

[0256] In one exemplary ELISA, the anti-estrogen receptor alpha antibodies of the invention are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition suspected of containing the wild-type and/or mutant estrogen receptor alpha protein antigen, such as a clinical sample, is added to the wells. After binding and/or washing to remove non-specifically bound immune complexes, the bound wild-type and/or mutant estrogen receptor alpha protein antigen may be detected. Detection is generally achieved by the addition of another anti-estrogen receptor alpha antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second anti-estrogen receptor alpha antibody, followed by the addition of a third antibody that has binding affinity for the second antibody, with the third antibody being linked to a detectable label.

[0257] In another exemplary ELISA, the samples suspected of containing the wild-type and/or mutant estrogen receptor alpha protein antigen are immobilized onto the well surface and/or then contacted with the anti-estrogen receptor alpha antibodies of the invention. After binding and/or washing to remove non-specifically bound immune complexes, the bound anti-estrogen receptor alpha antibodies are detected. Where the initial anti-estrogen receptor alpha antibodies are linked to a detectable label, the immune complexes may be detected directly. Again, the immune complexes may be detected using a second antibody that has binding affinity for the first anti-estrogen receptor alpha antibody, with the second antibody being linked to a detectable label.

[0258] Another ELISA in which the wild-type and/or mutant estrogen receptor alpha proteins, polypeptides and/or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies against wild-type or mutant estrogen receptor alpha protein are added to the wells, allowed to bind, and/or detected by means of their label. The amount of wild-type or mutant estrogen receptor alpha protein antigen in an unknown sample is then determined by mixing the sample with the labeled antibodies against wild-type and/or mutant estrogen receptor alpha before and/or during incubation with coated wells. The presence of wild-type and/or mutant estrogen receptor

alpha protein in the sample acts to reduce the amount of antibody against wild-type or mutant estrogen receptor alpha protein available for binding to the well and thus reduces the ultimate signal. This is also appropriate for detecting antibodies against wild-type or mutant estrogen receptor alpha protein in an unknown sample, where the unlabeled antibodies bind to the antigen-coated wells and also reduces the amount of antigen available to bind the labeled antibodies.

[0259] Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating and binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described below.

[0260] In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein or solutions of milk powder. The coating allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

[0261] In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, and a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or a third binding ligand.

[0262] "Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and/or antibodies with solutions such as BSA, bovine gamma globulin (BGG) or phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background.

[0263] The "suitable" conditions also mean that the incubation is at a temperature or for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours or so, at temperatures preferably on the order of 25°C to 27°C, or may be overnight at about 4°C or so.

[0264] Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immune complexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immune complexes may be determined.

[0265] To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact or incubate the first and second immune complex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under conditions that favor the development of further immune complex formation (*e.g.*, incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

[0266] After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, *e.g.*, by incubation with a chromogenic substrate such as urea, or bromocresol purple, or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), or H<sub>2</sub>O<sub>2</sub>, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generated, *e.g.*, using a visible spectra spectrophotometer.

## B. Immunohistochemistry

[0267] The antibodies of the present invention may also be used in conjunction with both fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks prepared for study by immunohistochemistry (IHC). The method of preparing tissue blocks from these particulate specimens has been successfully used in previous IHC studies of various prognostic factors, and/or is well known to those of skill in the art (Brown *et al.*, 1990; Abbondanzo *et al.*, 1990; Allred *et al.*, 1990).

[0268] Briefly, frozen-sections may be prepared by rehydrating 50 ng of frozen "pulverized" tissue at room temperature in phosphate buffered saline (PBS) in small plastic capsules; pelleting the particles by centrifugation; resuspending them in a viscous embedding medium (OCT); inverting the capsule and/or pelleting again by centrifugation; snap-freezing in -70°C isopentane; cutting the plastic capsule and/or removing the frozen cylinder of tissue;

securing the tissue cylinder on a cryostat microtome chuck; and/or cutting 25-50 serial sections.

[0269] Permanent-sections may be prepared by a similar method involving rehydration of the 50 mg sample in a plastic microfuge tube; pelleting; resuspending in 10% formalin for 4 hours fixation; washing/pelleting; resuspending in warm 2.5% agar; pelleting; cooling in ice water to harden the agar; removing the tissue/agar block from the tube; infiltrating and/or embedding the block in paraffin; and/or cutting up to 50 serial permanent sections.

### C. Immunodetection Kits

[0270] In still further embodiments, the present invention concerns immunodetection kits for use with the immunodetection methods described above. As the estrogen receptor alpha antibodies are generally used to detect wild-type and/or mutant estrogen receptor alpha proteins, polypeptides and/or peptides, or to detect the A908G mutation in estrogen receptor nucleic acid sequence, the antibodies will preferably be included in the kit. However, kits including both such components may be provided. The immunodetection kits will thus comprise, in suitable container means, a first antibody that binds to a wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or peptide, and/or optionally, an immunodetection reagent and/or further optionally, a wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or peptide.

[0271] In preferred embodiments, monoclonal antibodies will be used. In certain embodiments, the first antibody that binds to the wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or peptide may be pre-bound to a solid support, such as a column matrix and/or well of a microtitre plate.

[0272] The immunodetection reagents of the kit may take any one of a variety of forms, including those detectable labels that are associated with and/or linked to the given antibody. Detectable labels that are associated with and/or attached to a secondary binding ligand are also contemplated. Exemplary secondary ligands are those secondary antibodies that have binding affinity for the first antibody.

[0273] Further suitable immunodetection reagents for use in the present kits include the two-component reagent that comprises a secondary antibody that has binding affinity for the first antibody, along with a third antibody that has binding affinity for the second antibody, the third antibody being linked to a detectable label. As noted above, a

number of exemplary labels are known in the art and/or all such labels may be employed in connection with the present invention.

[0274] The kits may further comprise a suitably aliquoted composition of the wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or polypeptide, whether labeled and/or unlabeled, as may be used to prepare a standard curve for a detection assay. The kits may contain antibody-label conjugates either in fully conjugated form, in the form of intermediates, and/or as separate moieties to be conjugated by the user of the kit. The components of the kits may be packaged either in aqueous media and/or in lyophilized form.

[0275] The container means of the kits will generally include at least one vial, test tube, flask, bottle, syringe and/or other container means, into which the antibody may be placed, and/or preferably, suitably aliquoted. Where wild-type and/or mutant estrogen receptor alpha protein, polypeptide and/or peptide, and/or a second and/or third binding ligand and/or additional component is provided, the kit will also generally contain a second, third and/or other additional container into which this ligand and/or component may be placed. The kits of the present invention will also typically include a means for containing the antibody, antigen, and/or any other reagent containers in close confinement for commercial sale. Such containers may include injection and/or blow-molded plastic containers into which the desired vials are retained.

## **XVII. Two Hybrid Screen**

[0276] The term “two hybrid screen” as used herein refers to a screen to elucidate or characterize the function of a protein by identifying other proteins with which it interacts. The protein of unknown function, herein referred to as the “bait” is produced as a chimeric protein additionally containing the DNA binding domain of, for example, GAL4. Plasmids containing nucleotide sequences which express this chimeric protein are transformed into yeast cells, which also contain a representative plasmid from a library containing the respective GAL4 activation domain fused to different nucleotide sequences encoding different potential target proteins. If the bait protein physically interacts with a target protein, the GAL4 activation domain and GAL4 DNA binding domain are tethered and are thereby able to act conjunctively to promote transcription of a reporter gene. If no interaction occurs between the bait protein and the potential target protein in a particular cell, the GAL4 components remain separate and unable to promote reporter gene transcription on their own.

One skilled in the art is aware that different reporter genes can be utilized, including  $\beta$ -galactosidase, HIS3, ADE2, or URA3. Furthermore, multiple reporter sequences, each under the control of a different inducible promoter, can be utilized within the same cell to indicate interaction of the GAL4 components (and thus a specific bait and target protein). A skilled artisan is aware that use of multiple reporter sequences decreases the chances of obtaining false positive candidates. Also, alternative DNA-binding domain/activation domain components may be used, such as LexA. One skilled in the art is aware that any activation domain may be paired with any DNA binding domain so long as they are able to generate transactivation of a reporter gene. Furthermore, a skilled artisan is aware that either of the two components may be of prokaryotic origin, as long as the other component is present and they jointly allow transactivation of the reporter gene, as with the LexA system.

[0277] Two hybrid experimental reagents and design are well known to those skilled in the art (see *The Yeast Two-Hybrid System* by P. L. Bartel and S. Fields (eds.) (Oxford University Press, 1997), including the most updated improvements of the system (Fashena et al., 2000). A skilled artisan is aware of commercially available vectors, such as the Matchmaker™ Systems from Clontech (Palo Alto, CA) or the HybriZAP® 2.1 Two Hybrid System (Stratagene; La Jolla, CA), or vectors available through the research community (Yang et al., 1995; James et al., 1996). In alternative embodiments, organisms other than yeast are used for two-hybrid analysis, such as mammals (Mammalian Two Hybrid Assay Kit from Stratagene (La Jolla, CA)) or *E. coli* (Hu et al., 2000).

[0278] In an alternative embodiment, a two-hybrid system is utilized wherein protein-protein interactions are detected in a cytoplasmic-based assay. In this embodiment, proteins are expressed in the cytoplasm, which allows posttranslational modifications to occur and permits transcriptional activators and inhibitors to be used as bait in the screen. An example of such a system is the CytoTrap® Two-Hybrid System from Stratagene™ (La Jolla, CA), in which a target protein becomes anchored to a cell membrane of a yeast which contains a temperature sensitive mutation in the *cdc25* gene, the yeast homolog for hSos (a guanyl nucleotide exchange factor). Upon binding of a bait protein to the target, hSos is localized to the membrane, which allows activation of RAS by promoting GDP/GTP exchange. RAS then activates a signaling cascade which allows growth at 37°C of a mutant yeast *cdc25H*. Vectors (such as pMyr and pSos) and other experimental details are available for this system to a skilled artisan through Stratagene (La Jolla, CA). (See also, for example, U.S. Patent No. 5,776,689, herein incorporated by reference).



[0279] Thus, in accordance with an embodiment of the present invention, there is a method of screening for a peptide which interacts with ER $\alpha$  K303R polypeptide comprising introducing into a cell a first nucleic acid comprising a DNA segment encoding a test peptide, wherein the test peptide is fused to a DNA activation domain, and a second nucleic acid comprising a DNA segment encoding at least part of ER $\alpha$  K303R polypeptide, respectively, wherein the at least part of ER $\alpha$  K303R polypeptide, respectively, is fused to a DNA binding domain. Subsequently, there is an assay for interaction between the test peptide and the ER $\alpha$  K303R polypeptide or fragment thereof by assaying for interaction between the DNA activation domain and the DNA binding domain. In a preferred embodiment, the assay for interaction between the DNA binding and activation domains is activation of expression of  $\beta$ -galactosidase. In an alternative embodiment, the ER $\alpha$  K303R polypeptide is fused to the DNA activation domain and the test peptides are fused to the DNA binding domain.

### XVIII. Cancer

[0280] Tumors are notoriously heterogeneous, particularly in advanced stages of tumor progression (Morton *et al.*, 1993; Fidler and Hart, 1982; Nowell, 1982; Elder *et al.*, 1989; Bystryin *et al.*, 1985). Although tumor cells within a primary tumor or metastasis all may express the same marker gene, the level of specific mRNA expression can vary considerably (Elder *et al.*, 1989). It is, in certain instances, necessary to employ a detection system that can cope with an array of heterogeneous markers. In a specific embodiment, a marker for breast cancer comprises an A908G estrogen receptor alpha nucleic acid sequence or the K303R substitution to which it corresponds, or both.

[0281] Thus, while the present invention exemplifies various tumor suppressors as a markers, any marker that is correlated with the presence or absence of cancer may be used in combination with these markers to improve the efficacy of tumor detection and treatment. A marker, as used herein, is any proteinaceous molecule (or corresponding gene) whose production or lack of production is characteristic of a cancer cell. Depending on the particular set of markers employed in a given analysis, the statistical analysis will vary. For example, where a particular combination of markers is highly specific for melanomas or breast cancer, the statistical significance of a positive result will be high. It may be, however, that such specificity is achieved at the cost of sensitivity, *i.e.*, a negative result may occur even in the presence of melanoma or breast cancer. By the same token, a different combination may be very sensitive, *i.e.*, few false negatives, but has a lower specificity.

[0282] As new markers are identified, different combinations may be developed that show optimal function with different ethnic groups or sex, different geographic distributions, different stages of disease, different degrees of specificity or different degrees of sensitivity. Marker combinations also may be developed, which are particularly sensitive to the effect of therapeutic regimens on disease progression. Patients may be monitored after surgery, gene therapy, hyperthermia, immunotherapy, cytokine therapy, gene therapy, radiotherapy or chemotherapy, to determine if a specific therapy is effective.

[0283] There are many other markers that may be used in combination with these, and other, markers. For example,  $\beta$ -human chorionic gonadotropin ( $\beta$ -HCG) is produced by trophoblastic cells of placenta of pregnant woman and is essential for maintenance of pregnancy at the early stages (Pierce *et al.*, 1981; Talmadge *et al.*, 1984). b-HCG is known to be produced by trophoblastic or germ cell origin tumors, such as choriocarcinoma or testicular carcinoma cells (Madersbacher *et al.*, 1994; Cole *et al.*, 1983). Also ectopic expression of b-HCG has been detected by a number of different immunoassays in various tumors of non-gonadal such as breast, lung, gastric, colon, and pancreas, *etc.* (McManus *et al.*, 1976; Yoshimura *et al.*, 1994; Yamaguchi *et al.*, 1989; Marcillac *et al.*, 1992; Alfthan *et al.*, 1992). Although the function of b-HCG production in these tumors is still unknown, the atavistic expression of b-HCG by cancer cells and not by normal cells of non-gonadal origin suggests it may be a potentially good marker in the detection of melanoma and breast cancer (Hoon *et al.*, 1996; Sarantou *et al.*, 1997).

[0284] Another exemplary example of a marker is glycosyltransferase b-1, 4-N-acetylgalacto-saminytransferase (Ga1NAc). Ga1NAc catalyzes the transfer of N-acetylgalactosamine by b1(r) 4 linkage onto both gangliosides GM3 and GD3 to generate GM2 and GD2, respectively (Nagata *et al.*, 1992; Furukawa *et al.*, 1993). It also catalyzes the transfer of N-acetylgalactosamine to other carbohydrate molecules such as mucins. Gangliosides are glycosphingolipids containing sialic acids which play an important role in cell differentiation, adhesion and malignant transformation. In melanoma, gangliosides GM2 and GD2 expression, are often enhanced to very high levels and associate with tumor progression including metastatic tumors (Hoon *et al.*, 1989; Ando *et al.*, 1987; Carubia *et al.*, 1984; Tsuchida *et al.*, 1987a), although gangliosides are also expressed in melanoma, renal, lung, breast carcinoma cancer cells. The gangliosides GM2 and GD2 are immunogenic in humans and can be used as a target for specific immunotherapy such as human monoclonal antibodies or cancer vaccines (Tsuchida *et al.*, 1987b; Irie, 1985.)

[0285] Other markers contemplated by the present invention include cytolytic T lymphocyte (CTL) targets. MAGE-3 is a marker identified in melanoma cells and breast carcinoma. MAGE-3 is expressed in many melanomas as well as other tumors and is a (CTL) target (Gaugler *et al.*, 1994). MAGE-1, MAGE-2, MAGE-4, MAGE-6, MAGE-12, MAGE-Xp, and are other members of the MAGE gene family. MAGE-1 gene sequence shows 73% identity with MAGE-3 and expresses an antigen also recognized by CTL (Gaugler *et al.*, 1994). MART-1 is another potential CTL target (Robbins *et al.*, 1994) and also may be included in the present invention.

[0286] Preferred embodiments of the invention involve many different combinations of markers for the detection of cancer cells. Any marker that is indicative of neoplasia in cells may be included in this invention. A preferred marker is an A908G estrogen receptor alpha nucleic acid sequence and/or a K303R substitution in an estrogen receptor alpha nucleic acid sequence.

## **XIX. Pharmaceutical Preparations**

[0287] Pharmaceutical compositions of the present invention comprise an effective amount of one or more chimeric polypeptides or chimeric polypeptides and at least one additional agent dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one composition or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[0288] In some embodiments, an effective amount of a composition of the present invention, such as an antagonist to an estrogen receptor alpha K303R polypeptide, is administered to a cell. In other embodiments, a therapeutically effective amount of a composition of the present invention is administered to an individual for the treatment of disease. The term "effective amount" as used herein is defined as the amount of a composition of the present invention which is necessary to result in a physiological change in

the cell or tissue to which it is administered. The term "therapeutically effective amount" as used herein is defined as the amount of a composition of the present invention that eliminates, decreases, delays, or minimizes adverse effects of a disease, such as cancer. A skilled artisan readily recognizes that in many cases the composition may not provide a cure but may only provide partial benefit. In some embodiments, a physiological change having some benefit is also considered therapeutically beneficial. Thus, in some embodiments, an amount of a composition that provides a physiological change is considered an "effective amount" or a "therapeutically effective amount."

[0289] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, pp. 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the therapeutic or pharmaceutical compositions is contemplated.

[0290] The composition may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intranasally, intravitreally, intravaginally, intrarectally, topically, intratumorally, intramuscularly, intraperitoneally, subcutaneously, subconjunctival, intravesicularly, mucosally, intrapericardially, intraumbilically, intraocularly, orally, topically, locally, inhalation (e.g. aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, *via* a catheter, *via* a lavage, in cremes, in lipid compositions (e.g., liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

[0291] The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or

concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0292] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, the an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, etc., can be administered, based on the numbers described above.

[0293] In any case, the composition may comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[0294] The composition may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine.

[0295] In embodiments where the composition is in a liquid form, a carrier can be a solvent or dispersion medium comprising but not limited to, water, ethanol, polyol (*e.g.*, glycerol, propylene glycol, liquid polyethylene glycol, etc), lipids (*e.g.*, triglycerides, vegetable oils, liposomes) and combinations thereof. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin; by the maintenance of the required particle size by dispersion in carriers such as, for example liquid polyol or lipids; by the use of surfactants such as, for example hydroxypropylcellulose; or combinations thereof such methods. In many cases, it will be preferable to include isotonic agents, such as, for example, sugars, sodium chloride or combinations thereof.

[0296] In other embodiments, one may use eye drops, nasal solutions or sprays, aerosols or inhalants in the present invention. Such compositions are generally designed to be compatible with the target tissue type. In a non-limiting example, nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, in preferred embodiments the aqueous nasal solutions usually are isotonic or slightly buffered to maintain a pH of about 5.5 to about 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, drugs, or appropriate drug stabilizers, if required, may be included in the formulation. For example, various commercial nasal preparations are known and include drugs such as antibiotics or antihistamines.

[0297] In certain embodiments, the chimeric polypeptide is prepared for administration by such routes as oral ingestion. In these embodiments, the solid composition may comprise, for example, solutions, suspensions, emulsions, tablets, pills, capsules (*e.g.*, hard or soft shelled gelatin capsules), sustained release formulations, buccal compositions, troches, elixirs, suspensions, syrups, wafers, or combinations thereof. Oral compositions may be incorporated directly with the food of the diet. Preferred carriers for oral administration comprise inert diluents, assimilable edible carriers or combinations thereof. In other aspects of the invention, the oral composition may be prepared as a syrup or elixir. A syrup or elixir, and may comprise, for example, at least one active agent, a sweetening agent, a preservative, a flavoring agent, a dye, a preservative, or combinations thereof.

[0298] In certain preferred embodiments an oral composition may comprise one or more binders, excipients, disintegration agents, lubricants, flavoring agents, and combinations thereof. In certain embodiments, a composition may comprise one or more of the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or

combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, etc.; or combinations thereof the foregoing. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both.

[0299] Additional formulations which are suitable for other modes of administration include suppositories. Suppositories are solid dosage forms of various weights and shapes, usually medicated, for insertion into the rectum, vagina or urethra. After insertion, suppositories soften, melt or dissolve in the cavity fluids. In general, for suppositories, traditional carriers may include, for example, polyalkylene glycols, triglycerides or combinations thereof. In certain embodiments, suppositories may be formed from mixtures containing, for example, the active ingredient in the range of about 0.5% to about 10%, and preferably about 1% to about 2%.

[0300] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and/or the other ingredients. In the case of sterile powders for the preparation of sterile injectable solutions, suspensions or emulsion, the preferred methods of preparation are vacuum-drying or freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered liquid medium thereof. The liquid medium should be suitably buffered if necessary and the liquid diluent first rendered isotonic prior to injection with sufficient saline or glucose. The preparation of highly concentrated compositions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small area.

[0301] The composition must be stable under the conditions of manufacture and storage, and preserved against the contaminating action of microorganisms, such as bacteria

and fungi. It will be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein.

[0302] In particular embodiments, prolonged absorption of an injectable composition can be brought about by the use in the compositions of agents delaying absorption, such as, for example, aluminum monostearate, gelatin or combinations thereof.

## **XX. Methods of Making Transgenic Mice**

[0303] A particular embodiment of the present invention provides transgenic animals that comprise constructs having the A908G mutation. In another embodiment, the transgenic animal comprises a polynucleotide encoding an estrogen receptor alpha amino acid sequence comprising K303R. Transgenic animals expressing these mutations, recombinant cell lines derived from such animals, and transgenic embryos may be useful in methods for screening for and identifying agents that interact with the estrogen receptor alpha, or affect breast tissue health. .

[0304] In a general aspect, a transgenic animal is produced by the integration of a given transgene into the genome in a manner that permits the expression of the transgene. Methods for producing transgenic animals are generally described by Wagner and Hoppe (U.S. Patent 4,873,191; which is incorporated herein by reference), Brinster et al. 1985; which is incorporated herein by reference in its entirety) and in "Manipulating the Mouse Embryo; A Laboratory Manual" 2nd edition (eds., Hogan, Beddington, Costantini and Long, Cold Spring Harbor Laboratory Press, 1994; which is incorporated herein by reference in its entirety).

[0305] Typically, a gene flanked by genomic sequences is transferred by microinjection into a fertilized egg. The microinjected eggs are implanted into a host female, and the progeny are screened for the expression of the transgene. Transgenic animals may be produced from the fertilized eggs from a number of animals including, but not limited to reptiles, amphibians, birds, mammals, and fish.

[0306] DNA clones for microinjection can be prepared by any means known in the art. For example, DNA clones for microinjection can be cleaved with enzymes appropriate for removing the bacterial plasmid sequences, and the DNA fragments electrophoresed on 1% agarose gels in TBE buffer, using standard techniques. The DNA bands are visualized by staining with ethidium bromide, and the band containing the expression sequences is excised. The excised band is then placed in dialysis bags containing 0.3 M sodium acetate, pH 7.0. DNA is electroeluted into the dialysis bags, extracted with a



1:1 phenol:chloroform solution and precipitated by two volumes of ethanol. The DNA is redissolved in 1 ml of low salt buffer (0.2 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) and purified on an Elutip-D™ column. The column is first primed with 3 ml of high salt buffer (1 M NaCl, 20 mM Tris, pH 7.4, and 1 mM EDTA) followed by washing with 5 ml of low salt buffer. The DNA solutions are passed through the column three times to bind DNA to the column matrix. After one wash with 3 ml of low salt buffer, the DNA is eluted with 0.4 ml high salt buffer and precipitated by two volumes of ethanol. DNA concentrations are measured by absorption at 260 nm in a UV spectrophotometer. For microinjection, DNA concentrations are adjusted to 3 mg/ml in 5 mM Tris, pH 7.4 and 0.1 mM EDTA.

[0307] Other methods for purification of DNA for microinjection are described in Hogan et al. *Manipulating the Mouse Embryo* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1986), in Palmiter et al. *Nature* 300:611 (1982); in *The Qiagenologist, Application Protocols*, 3rd edition, published by Qiagen, Inc., Chatsworth, CA.; and in Sambrook et al. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989), all of which are incorporated by reference herein.

[0308] In an exemplary microinjection procedure, female mice six weeks of age are induced to superovulate with a 5 IU injection (0.1 cc, ip) of pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 hours later by a 5 IU injection (0.1 cc, ip) of human chorionic gonadotropin (hCG; Sigma). Females are placed with males immediately after hCG injection. Twenty-one hours after hCG injection, the mated females are sacrificed by CO<sub>2</sub> asphyxiation or cervical dislocation and embryos are recovered from excised oviducts and placed in Dulbecco's phosphate buffered saline with 0.5% bovine serum albumin (BSA; Sigma). Surrounding cumulus cells are removed with hyaluronidase (1 mg/ml). Pronuclear embryos are then washed and placed in Earle's balanced salt solution containing 0.5 % BSA (EBSS) in a 37.5°C incubator with a humidified atmosphere at 5% CO<sub>2</sub>, 95% air until the time of injection. Embryos can be implanted at the two-cell stage.

[0309] Randomly cycling adult female mice are paired with vasectomized males. FVB, C57BL/6 or Swiss mice or other comparable strains can be used for this purpose. Recipient females are mated at the same time as donor females. At the time of embryo transfer, the recipient females are anesthetized with an intraperitoneal injection of 0.015 ml of 2.5 % avertin per gram of body weight. The oviducts are exposed by a single midline dorsal incision. An incision is then made through the body wall directly over the oviduct. The ovarian bursa is then torn with watchmakers forceps. Embryos to be transferred are placed in DPBS (Dulbecco's phosphate buffered saline) and in the tip of a transfer pipet (about 10 to

12 embryos). The pipet tip is inserted into the infundibulum and the embryos transferred. After the transfer, the incision is closed by two sutures.

[0310] A skilled artisan is aware that transgenic mice are also commercially available, such as from Charles River Laboratories (Wilmington, MA).

### EXAMPLES

[0311] The following examples are offered by way of example, and are not intended to limit the scope of the invention in any manner.

#### EXAMPLE 1

##### MATERIALS AND METHODS--SAMPLE PREPARATION AND NUCLEOTIDE SEQUENCE ANALYSIS

[0312] Histologic slides from archival, clinical specimens were screened microscopically for evidence of hyperplasia. Microdissection of specimens was performed on 55 samples using serial sections from formalin-fixed, paraffin-embedded tissue blocks as described (O'Connell *et al.*, 1999). Briefly, alternative 3- and 10-um-thick sections were cut from the blocks and float mounted on glass slides. The 3-um-thick slides were stained with hematoxylin-eosin and examined under the light microscope to locate regions of normal and hyperplastic tissues; and these areas outlined with a felt-tipped pen. The marked slide was then used as a template to guide manual microdissection from the corresponding regions of the unstained 10-um-thick sections. It was possible to obtain distant normal tissue from 4 of the patients with hyperplasia. A skilled artisan recognizes that there are a variety of methods to isolate desired cells from undesired cells other than by manual manipulation or LCM. These include physical means of separating out undesired cells from desired cells, such as by centrifugation based on size, or centrifugation with magnetic beads attached to antibodies specific for desired and undesired cell types.

[0313] DNA was liberated from the microdissected specimens using a modification of the procedure of O'Connell *et al.* (1999). Genomic sequencing was then performed using PCR amplification of isolated DNA using ER primer 1 (nucleotides 1093-1112 (5' primer; 5'-CAAGCGCCAGAGAGATGATG-3'); SEQ ID NO:15) and ER primer 2 (nucleotides 1231-1250 (3' primer); 5'-ACAAGGCACTGACCATCTGG-3'; SEQ ID NO:16) of the ER gene (Greene *et al.*, 1996). An aliquot of this amplification was then used to perform single stranded PCR amplification using ER primer 3 (nucleotides 1221-1240

(3'primer); 5'-GACCATCTGGTCGGCCGTCA-3'; SEQ ID NO:17) of the ER gene. After precipitation of the single stranded PCR amplification product, dideoxysequence analysis was performed using ER primer 4 (nucleotides 1099-1119 (5'primer); CAGAGAGAATGATGGGGAGGG-3'; SEQ ID NO:18). In another embodiment, an alternative ER primer is used in lieu of ER primer 4, such as for nucleotides 1101-1130 (SEQ ID NO:35). Genomic DNA was isolated from normal blood samples of 80 healthy women, and utilized for genomic sequence analysis as described above. RNA was also isolated from four additional, frozen hyperplastic lesions, and utilized for RT/PCR amplification, cloning, and sequence analyses as described (Fuqua *et al.*, 1991).

## EXAMPLE 2

### MATERIALS AND METHODS--STABLE TRANSFECTION AND CELL GROWTH ANALYSES

[0314] The WT ER expression construct was prepared in the pcDNA1 vector as described previously (Fuqua *et al.*, 1995). Site directed mutagenesis of this construct was then utilized to generate the A908G transition and the entire coding sequence of ER was verified by dideoxysequence analysis in this clone. The generation of stable transfectants was performed as described by Oesterreich *et al.* (1993) using cotransfection with the G418-selectable expression vector pSVneo at a ration of 25:1 with the ER plasmids into MCF-7 breast cancer cells. To analyze for expression of both WT or Var sequences, Western blot analyses were performed using the 6F11 antibody (DaKO). Two to three-fold elevated levels of total ER protein were detected in the two WT ER and the three Var clones. In addition, RT/PCR amplification of cDNA from the transfectants (Fuqua *et al.*, 1991) followed by dideoxysequence analysis confirmed that exogenous WT and Var RNA were expressed in the stable transfectants. Furthermore, the relative levels of WT or Var sequences were determined by genomic sequence analysis as described above; the ER Var transfectants contained both WT nucleotide (A) and Var nucleotide (G) sequence in approximately equal ratios on the sequencing gels. For cell growth studies, cells were plated at a density of  $2 \times 10^4$  in media containing 10% charcoal-stripped, estrogen-free fetal calf serum and were either left untreated or treated with the indicated increasing estradiol concentrations of  $1 \times 10^{-12}$ ,  $1 \times 10^{-11}$ , or  $1 \times 10^{-9}$  M. The medium was replaced every 48 h and the cells were harvested and counted on days 2, 4, 6, and 8, respectively.

## EXAMPLE 3

## STATISTICAL METHODS

[0315] After taking logarithms to stabilize within group variances, as determined to be appropriate by Box-Cox analysis (Box and Cox, 1996), one-way analysis of variance was used to detect estrogen dose-related differences in growth on Day 8 (*i.e.* 0 versus  $10^{-12}$  M versus  $10^{-11}$  M versus  $10^{-9}$  M), and to detect differences among estrogen doses ( $10^{-12}$  M versus  $10^{-11}$  M versus  $10^{-9}$  M). The Student-Newman-Keuls multiple range test ( $\alpha = 0.02$ ) was used to determine which doses were different from each other. Analyses were preformed using SAS (V6.12, SAS Institute, Cary, NC).

## EXAMPLE 4

## MATERIALS AND METHODS--GST PULL-DOWN ASSAYS

[0316] Bacterial expression vectors for GST-wt ER and GST-mutant ER were constructed by PCR amplification of the hinge and hormone binding domains of wild-type ER  $\alpha$  and the A908G ER  $\alpha$  using a sense primer (nucleotides 756-775 and an antisense primer (nucleotides 1788-1769) (Greene *et al.*, 1996), and then cloning these products into the BamH1-EcoRI sites of pGEX-2kt GST gene fusion vector (Pharmacia). The GST-pull down assays were performed as described (Ding *et al.*, 1998) using recombinant SRC-2 (pSG5-human TIF-2) translated *in vitro* using the TNT coupled Reticulocyte Lysate System (Promega, Madison, WI), as well as recombinant SRC-1 and SRC-3. The reactions were allowed to bind the glutathione-Sepharose 4B beads (Pharmacia) for 1.5 h in the presence of increasing amounts of estradiol at 4°C. Samples were subsequently analyzed by SDS-polyacrylamide gel electrophoresis.

## EXAMPLE 5

ASSAY OF ESTROGEN RECEPTOR ALPHA SEQUENCE  
IN EARLY BREAST DISEASE

[0317] cDNA was prepared by reverse transcription of RNA from 4 typical hyperplasias of the breast, to assay for an altered ER in early breast disease, followed by polymerase chain reaction (PCR) amplification using primers specific for the entire coding domain of ER $\alpha$  (across nucleotides 1182 to 1234). Cloning and sequencing of ER was performed as described in Fuqua *et al.* (1991) except restriction sites were incorporated into

the primers to facilitate cloning into pGEM7zf (+) (Promega Corp., Madison, WI). Wildtype ER sequence was identified in two of these premalignant lesions (FIG. 2). However, in the other two lesions an ER  $\alpha$  variant was identified with an A to G base pair transition at nucleotide 908 (FIG. 2, top panel). This transition introduces a Lys to Arg substitution at residue 303 within exon 4, at the border between the hinge domain D and the beginning of the hormone-binding domain E of ER $\alpha$  (FIG. 2, bottom diagram). Even though this substitution represents a conservative amino acid change, the size of the study was enlarged, since the data indicates that the amino-terminal region of the ER $\alpha$  hormone-binding domain is important in the generation of a complete transcriptional response in cells (Pierrat *et al.*, 1994). Therefore, archival histological sections of 55 additional typical hyperplasias were microdissected, DNA was isolated, and direct genomic sequencing was performed using primers bordering ER $\alpha$  nucleotide 908. The same ER  $\alpha$  alteration in 18/55 of these additional premalignant lesions was identified. Thus, the A908G ER $\alpha$  alteration was present in a total of 20/59 (34%) of the hyperplasias examined.

[0318] DNA was prepared from normal breast epithelium adjacent to the hyperplastic lesion of those samples that contained the A908G ER $\alpha$  alteration. The ER $\alpha$  variant sequence was detected in the normal adjacent epithelium of some of these samples tested. Thus, the A908G ER  $\alpha$  transition is frequently present in premalignant lesions of the breast, and can occur in the adjacent normal-appearing breast epithelium.

## EXAMPLE 6

### THE A908G ER $\alpha$ MUTATION IS A SOMATIC MUTATION

[0319] To address whether the ER alteration might represent a somatic change in the breast, rather than a germ-line alteration or a naturally-occurring polymorphism within ER $\alpha$ , distant normal epithelium from 4 of the 20 patients with the A908G ER alteration in their hyperplastic lesion was microdissected. (Only 4 of the patients had sufficient normal distant tissue for analysis.) Manual microdissection on a light box under a dissecting microscope was performed to microdissect archival, formalin-fixed, paraffin-embedded tissue blocks and was precise enough to ensure at least 50% cellularity. DNA was liberated from the microdissected specimens and direct genomic sequence analysis performed. Genomic sequencing of one patient's samples is shown in FIG. 3. Variant A908G ER  $\alpha$  sequence was detected along with WT sequence in the normal adjacent DNA (N Adj.) and the typical

hyperplasia (TH) DNA from this patient, but the normal distant tissue (N Dis.) displayed only WT ER  $\alpha$  sequence. All 4 of the patients with the variant ER $\alpha$  sequence in their hyperplastic lesion exhibited WT sequence in their distant normal tissue. To further strengthen this observation, normal DNA was also examined by direct genomic sequencing of 80 blood samples collected from patients without breast disease. There was no detection of the ER $\alpha$  variant sequence in any of these normal samples. Therefore, the A908G ER $\alpha$  alteration is a somatic mutation appearing frequently in association with breast hyperplasia. Thus, just as LOH can occur in morphologically normal ductal epithelium adjacent to breast cancers (Deng *et al.*, 1996), and may therefore demarcate a localized region predisposed to the development of breast cancer, in a specific embodiment a somatic mutation in ER $\alpha$  within a localized region of normal breast epithelium defines a region of increased risk if the mutation confers a selective advantage to these cells.

#### EXAMPLE 7

##### THE A908G ER $\alpha$ MUTATION CONFERS SELECTIVE ADVANTAGE TO CELLS

[0320] The proliferative response to hormones in breast cancer cell transfectants containing the mutation was tested to determine if this ER mutation confers a selective advantage. A CMV-driven mammalian expression vector was prepared for WT ER $\alpha$  and utilized site-directed mutagenesis (Promega, Madison, WI) to generate the Lys303Arg substitution. The mutant expression vector was stably introduced into the ER-positive MCF-7 breast cancer cell line that normally expresses WT ER $\alpha$ . This cell line was chosen because it was determined that WT ER $\alpha$  was expressed along with the mutant in the original 2/4 typical hyperplastic lesions which were examined. As a control, the expression vector was also stably transfected alone into MCF-7 cells. Transfected clones were then cultivated in estrogen-depleted medium (-E<sub>2</sub>) or medium supplemented with increasing amounts of estradiol ( $10^{-12}$  to  $10^{-9}$ M). Both non-transfected MCF-7 cells (FIG. 4, panel A) and vector-alone transfected cells (panel B) exhibited typical estrogen dose response growth curves. Minimal cell growth stimulation was seen with  $10^{-12}$ M estradiol in these cells. Because it was possible that overexpression of the receptor alone might stimulate the growth of these cells, MCF-7 cells were also transfected with the expression vector for WT ER $\alpha$ , but their estrogen dose response curves (FIG. 4, panels C and D) were not different from the controls (Oesterreich *et al.*, 1993). In contrast, three independent clones expressing the ER $\alpha$  mutation responded to extremely low levels of hormone ( $10^{-12}$  M) (FIG. 4, panels E, F, and G) with

nearly the same highly proliferative response seen at the highest concentration of estradiol used ( $10^{-9}$  M).

[0321] Using analysis of variance (Box and Cox, 1996), it was determined that these were highly significant estrogen dose responses in the MCF-7, vector-alone transfected, and WT ER $\alpha$ -transfected cells ( $p=0.001$ ), but that there was little or no difference in response to differing concentrations of estradiol in each of the three mutant ER $\alpha$ -transfected clones ( $p=0.41$ ,  $0.015$ , and  $0.09$ , respectively, for clones E, F, and G). The growth-stimulatory effects of low levels of hormone in cells expressing the ER $\alpha$  mutation were even more evident when doubling times were calculated from the growth curves. For example, the doubling time for MCF-7 cells in  $10^{-12}$  or  $10^{-9}$  M estradiol is 2.2 vs. 1.3 days, respectively. The doubling times for cells expressing the ER $\alpha$  mutant is the same (1.3 days) at either  $10^{-12}$  or  $10^{-9}$  M of hormone. Thus, the expression of the ER $\alpha$  mutation confers a hypersensitivity to estrogen with an ability to be maximally stimulated in response to physiological levels ( $10^{-12}$  to  $10^{-11}$  M) of hormone. Thus, the A908G ER $\alpha$  mutation is a gain-of-function mutation that could have a significant biological role in early breast disease.

[0322] In one embodiment, one mechanism by which the ER $\alpha$  mutation confers hypersensitivity to low levels of hormone would be an increased binding affinity for estradiol. However, no differences in estradiol affinity were detected between the WT ER $\alpha$  and the A908G ER $\alpha$  mutation using saturation binding Scatchard analyses, nor were there differences in affinity for the antiestrogen tamoxifen.

[0323] In an alternative embodiment, one mechanism by which the ER $\alpha$  mutation confers hypersensitivity to low levels of hormone might be altered affinity for ER co-regulators. It is now understood that many of the cell-type and tissue-specific effects of ER $\alpha$  are dependent on the cellular pool of co-regulatory factors that bind to and influence its transcriptional activity (reviewed in Horowitz *et al.*, 1997), many of which act as signaling intermediates between the ER and the general transcriptional machinery, or directly have enzymatic activities such as histone acetyltransferase activity. The A908G ER $\alpha$  mutation occurs in a region implicated in binding to certain of these co-regulatory proteins, such as L7/SPA (Jackson *et al.*, 1997) and the SRC-1 family of co-activators (Onate *et al.*, 1998). For example, efficient interaction of SRC-1 with the progesterone receptor hormone-binding domain requires the presence of hinge sequences (Onate *et al.*, 1998). Thus, the ability of WT and mutant ER $\alpha$  to interact with SRC-2 (TIF-2) (Voegel *et al.*, 1996), a member of the

SRC-1 family, was compared using *in vitro* GST pull-down assays (Ding *et al.*, 1998). GST-WT ER $\alpha$  and GST-ER $\alpha$  mutant fusion constructs containing the hinge and hormone binding domains were prepared. Full-length SRC-1, SRC-2 and SRC-3 were synthesized *in vitro* in the presence of [<sup>35</sup>S]methionine and then tested for specific hormone-dependent binding to the immobilized GST-ER fusion proteins (FIG. 5) by incubating with Sepharose beads containing immobilized GST, GST-WT ER, and GST-A908G mutant ER with or without estradiol. Bound SRC-1, SRC-2 and SRC-3 were eluted and observed by SDS-PAGE and autoradiography. Input SRC-1, SRC-2 and SRC-3 are shown (10%), as is nonspecific GST binding in the absence of estradiol. Increasing levels of estradiol used were: 4 X 10<sup>-8</sup>, 5 X 10<sup>-8</sup>, 6 X 10<sup>-8</sup>, 7 X 10<sup>-8</sup>, and 1 X 10<sup>-6</sup>M. Both receptors bound SRC-1, SRC-2 and SRC-3 in the presence (10<sup>-6</sup> M), but not the absence of estradiol. However, the mutant required much less hormone for efficient binding. Even at the lowest estradiol concentration tested, 4 X 10<sup>-8</sup>M, the mutant ER efficiently bound SRC-2 and SRC-3, whereas WT ER $\alpha$  exhibited negligible binding at this concentration. The mutant ER also bound SRC-1 co-activator, although not to the same extent as SRC-2 and SRC-3. This data indicates that the Lys303Arg substitution enhances SRC-1, SRC-2 and SRC-3 binding by lowering the concentration of hormone required to facilitate the formation of the co-activator:ER hydrophobic groove binding surface (Shiau *et al.*, 1998) within the ER hinge/ligand binding domain. In another embodiment, an additional mechanism includes this residue in the ER as a site for acetylation. An Arg substitution at this site could render it incapable of being acetylated, and/or the substitution could reduce the net negative charge if surrounding Lys residues in the ER are indeed acetylated. Altered co-activator binding has also been reported for a Tyr537Asn ER $\alpha$  mutation (Tremblay *et al.*, 1998) that was identified in a metastatic bone lesion from a breast cancer patient (Zhang *et al.*, 1997). Thus, it is important that both of these *in vivo* ER $\alpha$  mutations drastically affect the ability of the receptor to bind to co-regulatory proteins.

[0324] A skilled artisan recognizes that there are alternative methods in the art to testing for acetylation in addition to immunodetection methods.



## EXAMPLE 8

SINGLE STRAND CONFORMATION POLYMORPHISM  
(SSCP) ANALYSIS OF ER MUTATION

[0325] A skilled artisan recognizes that there are multiple methods known in the art to identify a mutation, including SSCP. Additional clinical samples were examined by manually microdissecting permanent sections of 10 typical hyperplasias. Manual microdissection on a light box under a dissecting microscope was performed to microdissect archival, formalin-fixed, paraffin-embedded tissue blocks and was precise enough to ensure at least 50% cellularity. DNA was liberated from the microdissected specimens as described (Fuqua *et al.*, 1991) and SSCP analysis performed (Orita *et al.*, 1989) using primers spanning across ER nucleotide 908 (FIG. 6). SSCP was performed as previously described (Elledge *et al.*, 1993) except ER primers were used for PCR amplification (nucleotides 1093-1112 (5' primer; SEQ ID NO:15) and 1231-1250 (3' primer; SEQ ID NO:16) of the ER gene (Greene *et al.*, 1986). The gels were electrophoresed in 0.5X TBE at room temperature for 14h.. To be scored as having an alteration, a DNA sample had to produce an abnormal SSCP pattern using separate DNA aliquots and amplified on different days with negative controls.

[0326] Five of the hyperplasias (samples 2, 4, 5, 7, and 8) displayed band mobilities which were identical to those of the complementary strands of the PCR fragment from the WT ER control DNA. However, in five of the hyperplasias (samples 1, 3, 6, 9, and 10) four bands could be detected. These results indicated that the DNA from these later five hyperplasias had two different ER alleles, one WT and the other migrating identical with the mutant (Mut) ER allele. Further proof that these faster migrating bands contained the A908G transition was obtained by cutting the region corresponding to the Mut band from the dried gel, cloning the fragment, and dideoxysequence analyzing to confirm.

## EXAMPLE 9

## OLIGONUCLEOTIDE MISMATCH MUTATION DETECTION

[0327] A sensitive oligonucleotide mismatch hybridization method (Moul *et al.*, 1992) was used to detect the ER alleles in a cancer patient. In addition, laser capture microdissection was utilized to more precisely enrich for the specific lesions present concomitantly in this patient.

[0328] A nested PCR amplification procedure was used to amplify the laser capture microdissected material (Bonner, 1997) where the outside primers correspond to

those used in the SSCP analysis described above in a 30 $\mu$ l reaction volume, and then 1.5  $\mu$ l of this was then reamplified with ER primer sequences corresponding to nucleotides 1101-1130 (5') and 1220-1239 (3') of the ER gene (Greene *et al.*, 1986). The samples were then denatured in 0.4 M NaOH, 25 mM EDTA at 95°C, then neutralized with 1 M Tris-HCl pH 7.4 before slotting on the nylon membranes. Oligonucleotide probes corresponding to the WT (SEQ ID NO:33; 5'-GCTCTAAGAAGAAGACAGCCTG-3') or Mutant (SEQ ID NO:34; 5'-GCTCTAAGAGGAACAGCCTG-3') (corresponding to nucleotides 1191 to 1210 of the ER gene (Greene *et al.*, 1986)) were end-labeled with T4 kinase. The membrane was prehybridized in 5X SSPE, 0.5% SDS, 5X Denhardt's and washed at 60°C 2X SSPE, 0.1% SDS followed by a wash at 68°C in 5XSSPE. 0.1% SDS. Control WT or Mut plasmid DNAs were also amplified, slotted, and hybridized as positive controls for hybridization; samples without added DNA were included as negative controls during amplification.

[0329] The variant sequence was detected in the normal adjacent breast epithelium (AB), the hyperplastic lesion (H), and one ductal carcinoma in situ (DCIS) lesion using an oligonucleotide probe specific for the variant, but not in normal skin (NS), normal distant breast epithelium (DB), or another independent DCIS lesion in this patient (FIG. 7, right panel). Both WT (FIG. 7, left panel) and mutant ER alleles were present in this patient.

#### EXAMPLE 10

##### INCIDENCE OF THE A908G MUTATION IN INVASIVE BREAST CANCERS

[0330] In a specific embodiment of the present invention, breast cancer samples from invasive breast tumors are assayed by standard methods, such as those described herein, for the A908G mutation in estrogen receptor alpha nucleic acid sequence. A skilled artisan recognizes that there are presently two types of invasive breast cancer: Node-negative and Node-positive. In approximately half of women with invasive breast cancer, the lymph nodes are invaded (Node-positive), and there are also micrometastases elsewhere within the body. In approximately half of women with invasive breast cancer, the cancer has not spread to the lymph nodes.

	Ca. from Node-negative women	Ca. from Node-positive women
Wild-type	16	4
Mutant	10	23

(p= 0.00062 Fisher's Exact Test, two sided)

[0331] Therefore, the frequency of the mutation in invasive breast tumors = 33/53 = 62%. Thus, the A908G mutation is identified in both Node-negative and Node-positive invasive breast cancers.

### EXAMPLE 11

#### SCREENING FOR ANTAGONISTS AND AGONISTS OF ER $\alpha$ K303R POLYPEPTIDE

[0332] In some embodiments of the present invention, candidates for drugs are screened which are useful for treatment of a breast cancer related to the A908G mutation in ER $\alpha$  polynucleotide and/or the ER $\alpha$  K303R polypeptide which it encodes. In specific embodiments, antagonists or agonists are screened for which affect the activity of the ER $\alpha$  K303R polypeptide.

[0333] A skilled artisan recognizes that a variety of methods known in the art are available to screen for antagonists or agonists of ER $\alpha$  K303R polypeptide. For example, transfection assays are utilized (such as described in Barkhem *et al.* (1997); Cowley *et al.* (1997); and Sun *et al.* (1999), all of which are incorporated by reference herein in their entirety) wherein a cell is transiently or stably transfected with an expression vector comprising the ER form to be tested against, a reporter expression construct operably linked to at least one estrogen response element, such as 5'-AGGTCA-3' (SEQ ID NO:36); 5'-TGACCT-3' (SEQ ID NO:37); 5'-GGTCAnnnTGACC-3' (SEQ ID NO:38); 5'-AATCAnnnTGACT-3' (SEQ ID NO:39); 5'-GGTCA-3' (SEQ ID NO:40); 5'-TGGTC-3' (SEQ ID NO:41); 5'-TGACC-3' (SEQ ID NO:42); 5'-ATTCGATCAGGGCGGGGCGAGC-3' (from SP1; SEQ ID NO:43); 5'-GGGCA(N)<sub>16</sub>GGCGGG-3' (c-myc; SEQ ID NO:44); 5'-GGTCA(N)<sub>21</sub>GGCGG-3' (ckb; SEQ ID NO:45); 5'-GGGCCGGG(N)<sub>10</sub>GGTCA-3' (cathepsin D; SEQ ID NO:46); 5'-GGGCA-3' (hsp27; SEQ ID NO:47); 5'-GGTAA-3' (cathepsin D; SEQ ID NO:48); 5'-GGTCA(N)3TGCCC-3' (uteroglobin; SEQ ID NO:49); 5'-GGGGCGTGG-3' (c-fos; SEQ ID NO:22); 5'-CCGCCCC-3' (e2f; SEQ ID NO:26); 5'-TGA(C/G)TCA-3' (AP1; SEQ ID NO:8). A compound to be tested is administered to the cell, and the expression level of the reporter expression construct is assayed in the presence of the test compound and compared to expression levels in its absence. A test compound which downregulates expression of the reporter polynucleotide is considered an antagonist, and a

test compound which upregulates expression of the reporter polynucleotide is considered an agonist.

[0334] In alternative embodiments for drug/antagonist/agonist screening, a two hybrid assay is performed, such as is described in Slentz-Kesler *et al.* (2000), incorporated by reference herein in its entirety. In a specific embodiment, a polynucleotide encoding the ER $\alpha$  K303R polypeptide as a fusion polypeptide with a DNA binding domain is transformed into a yeast or mammalian cell. The population of corresponding yeast or mammalian cells further comprise a library of expression vectors producing chimeric polypeptides comprising a DNA activation and a library candidate. Interaction of the ER $\alpha$  K303R polypeptide with a particular library candidate is visualized by assaying expression of a reporter sequence expression influenced by the interaction of the corresponding DNA activation and binding domains. A skilled artisan recognizes that multiple DNA activation and binding domains are available, including GAL4 or LexA. Also, controls are performed to eliminate any false positives.

[0335] In another embodiment to identify and design drugs for ER $\alpha$  K303R polypeptide-associated breast cancer, particularly antagonists and agonists, a phage peptide display assay is employed, such as is described in Sparks *et al.* in Phage Display of Peptides and Proteins, A Laboratory Manual (Academic, San Diego), incorporated by reference herein. In this embodiment, an affinity-tagged labeled ER $\alpha$  K303R polypeptide is exposed to a nitrocellulose membrane comprising bacteriophage plaques each of which comprise a peptide. Binding of the ER $\alpha$  K303R polypeptide to the peptide is assayed, and the resultant peptides are identified. In some embodiments, the affinity selection of the phage-displayed peptide libraries is conducted on the ER $\alpha$  K303R polypeptide in different conditions, such as in an apo form, ligand-bound form, and so forth. The resultant peptides are analyzed, allowing rational drug design to ensue based on the analysis.

[0336] In an additional embodiment, other methods are known to evaluate the effects of an antagonist vs. an agonist of a receptor-binding substance on a selected type of cells containing an endogenous intra-cellular hormone receptor, such as is described in U.S. Patent No. 5,578,445, incorporated by reference herein. Therein, an *in vitro* method is disclosed wherein a test substance and a reference substance, known to be either an antagonist or an agonist of the receptor, is incubated with cells, and the magnitude of the selected cellular response resulting from the hormone/receptor interaction is analyzed.

[0337] In another embodiment, drug candidates/ antagonists/ agonists for ER $\alpha$  K303R polypeptide are analyzed by mass spectrometry (Witkowska *et al.*, 1997) or by X-ray crystallography (Shiau *et al.*, 1998), both of which are incorporated by reference herein in their entirety. A skilled artisan recognizes that the National Center for Biotechnology Information provides a structural database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed>) containing many protein structures, including estrogen receptor. Analyses of the ER $\alpha$  K303R polypeptide by these methods provide significant structural detail so that, for example, an antagonist to fit a particular structural domain can be designed. In one embodiment, X-ray crystallography is performed on the ER $\alpha$  K303R polypeptide bound to a specific ligand, such as estradiol, tamoxifen, raloxifene, droloxigene, GW 5638, idoxifene, CP336156, or LY353381. Such an analysis facilitates design of a drug which will antagonize the activity of the ER $\alpha$  K303R polypeptide, such as for the treatment of breast cancer. For mass spectrometry methods to facilitate drug screen/antagonist/agonist analysis, methods may be employed which are similar to Witkowska *et al.* (1997), wherein structural comparisons were made between two structurally similar compounds. In a particular embodiment, the mass spectrometry analysis provides information on binding sites for cofactors.

[0338] In one embodiment of the present invention, there is a method of designing an agent which affects the activity of an estrogen receptor alpha K303R polypeptide, comprising determining the crystal structure of a purified estrogen receptor alpha K303R polypeptide; and analyzing a model of the crystal structure, wherein the agent is designed based on the analysis.

[0339] In another embodiment of the present invention there is a method of designing an agent which affects the activity of an estrogen receptor alpha K303R polypeptide, comprising determining the crystal structure of a purified estrogen receptor alpha K303R polypeptide in the presence of a compound which interacts with the estrogen receptor alpha K303R polypeptide; and analyzing a model of the crystal structure, wherein the agent is designed based on the analysis. In a specific embodiment, the analyzing step comprises computer modeling. In another specific embodiment, the crystal structure is determined in the presence of an estrogen receptor ligand.

[0340] In an additional embodiment of the present invention, there is a method of designing an agent which affects the activity of an estrogen receptor alpha K303R polypeptide, comprising analyzing the structure of the polypeptide by mass spectrometry,

wherein the structure of the polypeptide suggests the design of the activity-affecting agent. In a specific embodiment, the activity-affecting agent is an antagonist. In another specific embodiment, the activity-affecting agent is an agonist.

## EXAMPLE 12

### SIGNIFICANCE OF THE PRESENT INVENTION

[0341] In summary, it is shown that a large proportion of premalignant breast hyperplasias express an altered ER $\alpha$  that is hypersensitive to the effects of estrogen. Furthermore, the alteration results from a somatic mutation in the breast with this mutation affecting the ability of the receptor to bind to the SRC-1, SRC-2, and SRC-3 co-activators. There is an increasing body of evidence, both epidemiological (Dupont and Page, 1985) and molecular (O'Connell *et al.*, 1998), suggesting that these premalignant lesions are both risk factors and direct precursors of invasive breast cancer. However, hyperplasias are relatively common in the breast, and only a small fraction of them will progress to cancer. Prior to the methods and compositions of the present invention, those in the art have been unable to differentiate which of these lesions are genetically stable, or the biological differences driving some of them to progress. An ER $\alpha$  mutation that confers a proliferative advantage, such as hypersensitivity to hormone, in a specific embodiment provides a favorable cellular environment accelerating the accumulation of additional genetic events important for tumor progression.

[0342] Premalignant breast lesions are microscopic masses with a positive growth imbalance, and the hypersensitive ER $\alpha$  mutation is likely an important factor contributing to this imbalance. Hormone levels normally fluctuate during the menstrual cycle in premenopausal women, and levels are considerably lower in postmenopausal women. In one embodiment, an ER mutation hypersensitive to estradiol provides a continuous mitogenic stimulus to the breast epithelium even during phases of low circulating hormone, especially in postmenopausal women, thus elevating their risk for breast cancer. Thus, in a preferred embodiment, there is a correlation between risk for breast cancer and expression of this ER $\alpha$  mutation, which will allow genetic analysis for the mutation in premalignant lesions to be crucial to identify patients who would benefit from preventive measures.

**EXAMPLE 13****DUCTAL HYPERPLASIAS IN K303R TRANSGENIC MICE**

[0343] Transgenic mice expressing the K303R mutation were generated by standard means in the art. The mice at the time of filing of the nonprovisional application have matured to 18 months, and they have developed ductal hyperplasias (FIG. 8, panels A through D). Nontransgenic mammary glands are shown in panels 8E and 8F. The H&E-stained histological sections shown in panels 8A and 8B clearly demonstrate the development of ductal hyperplasias in the transgenic mice with luminal epithelial cells beginning to stratify in the ductal lumen in the mammary glands. Panel 8B shows a duct whose lumen is completely filled with epithelial cells. In a specific embodiment of the present invention, the hypersensitive ER mutation provides a proliferative advantage, especially by providing a continuous mitogenic stimulus to the epithelium even in an environment of low circulating hormones, such as these virgin mice.

[0344] Ductal hyperplasias are composed of both an increase in the number of epithelial cell layers within the duct (shown in panel 8C), as well as an increase in the number of small ducts within a given area (shown in panel 8D). These increases in the transgenic animals are more clearly observed when one compares the histological sections from nontransgenic mammary glands (shown in panels 8E and 8F).

[0345] FIG. 9 shows that the K303R transgenic animals have increased proliferation as compared to nontransgenic animals in the ductal epithelium. Proliferation was measured by immunohistochemistry with an antibody to phosphorylated histone H1b, a surrogate marker of S-phase.

[0346] Thus, the data in FIGS. 8 and 9 show that expression of the K303R mutation, which was originally identified in human breast hyperplastic lesions, is indeed an important factor contributing to abnormal ductal growth and the development of proliferating ductal hyperplasias.

**REFERENCES**

[0347] All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference herein.

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[0348] One skilled in the art readily appreciates that the patent invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. Mutations, kits, sequences, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the pending claims.